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The biological activity of a new class of mechanism-based serine protease inhibitors

H.M.M. Bastiaans

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THE BIOLOGICAL ACTIVITY OF A NEW CLASS OF MECHANISM-BASED SERINE PROTEASE INHIBITORS

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VRIJE UNIVERSITEIT

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Aan mijn ouders

voor Ellen

'With it share of glorious moments, setbacks, and frustrations, total synthesis can be compared to the game of chess. The object of this game is to capture the king by a series of allowed moves played out in such a combination and order as to outmaneuver the opponent.'

K.C. Nicolaou

The investigations described in this thesis were performed at the Division of Medicinal Chemistry of the Leiden/Amsterdam Center for Drug Research (LACDR) and at the Department of Organic and Inorganic Chemistry at the Vrije Universiteit, Amsterdam, The Netherlands.
The enzyme inhibition studies described in Chapter Seven were carried out at the Department of Vascular Pharmacology, Scientific Development Group, N.V. Organon, Oss, The Netherlands.

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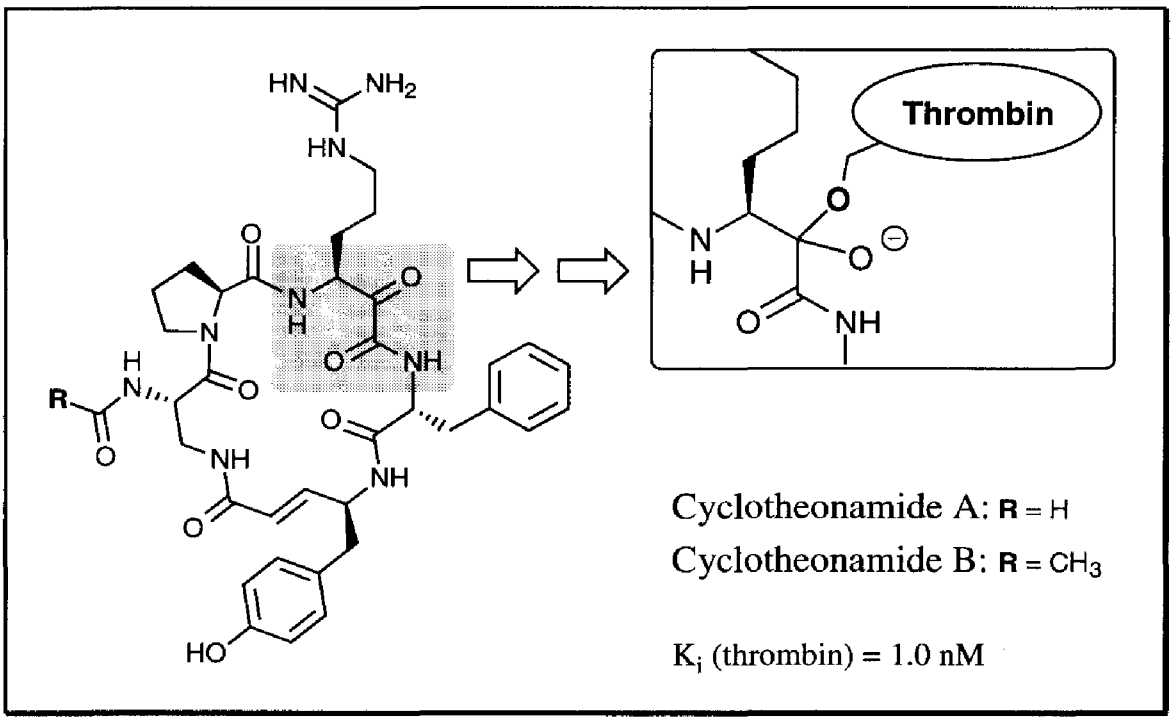
CHAPTER ONE

General Introduction



Abstract

In this introductory chapter, a brief survey of the hemostatic system and the central role of thrombin will be given. Inhibition of thrombin offers an important treatment for diseases and pathological conditions where thrombosis can cause life threatening situations. Several thrombin inhibitors, of which some are currently used in the treatment of thrombosis, are discussed. Nevertheless, the development of more potent, more selective and orally active thrombin inhibitors is still a challenging objective.

Cyclotheonamides A/B, potent thrombin inhibitors recently isolated from a marine sponge, may offer a basis for the development of a novel class of thrombin inhibitors. A discussion of the unique structure and biological activity of these natural products is found at the end of this chapter.

1.1. Introduction

After injury of the vessel wall, blood coagulation is activated and initiates the formation of an insoluble polymer which results in a blood clot (hemostatic plug) to prevent further bleeding. Once coagulation is initiated, the hemostatic system first localizes the clot at the site of the injury, eventually terminates coagulation and finally removes the clot (by fibrinolysis) after it has served its purpose. The fibrinolytic system also prevents formation of intravascular clots outside the traumatized area.
When the complex system of coagulation and fibrinolysis is out of balance, life threatening situations may occur through excessive bleeding or by thrombosis which can result in a stroke, myocardial infarct or thromboembolism.[1]

In Section 1.2 the process of hemostasis will be outlined in a concise fashion to merely illustrate the action of several classes of antithrombotic agents discussed in Section 1.3. A more elaborate discussion on hemostasis and thrombosis can be found in several comprehensive reviews and recent books [Section 1.6: General References].

1.2. Hemostasis

1.2.1. Fibrin cascade

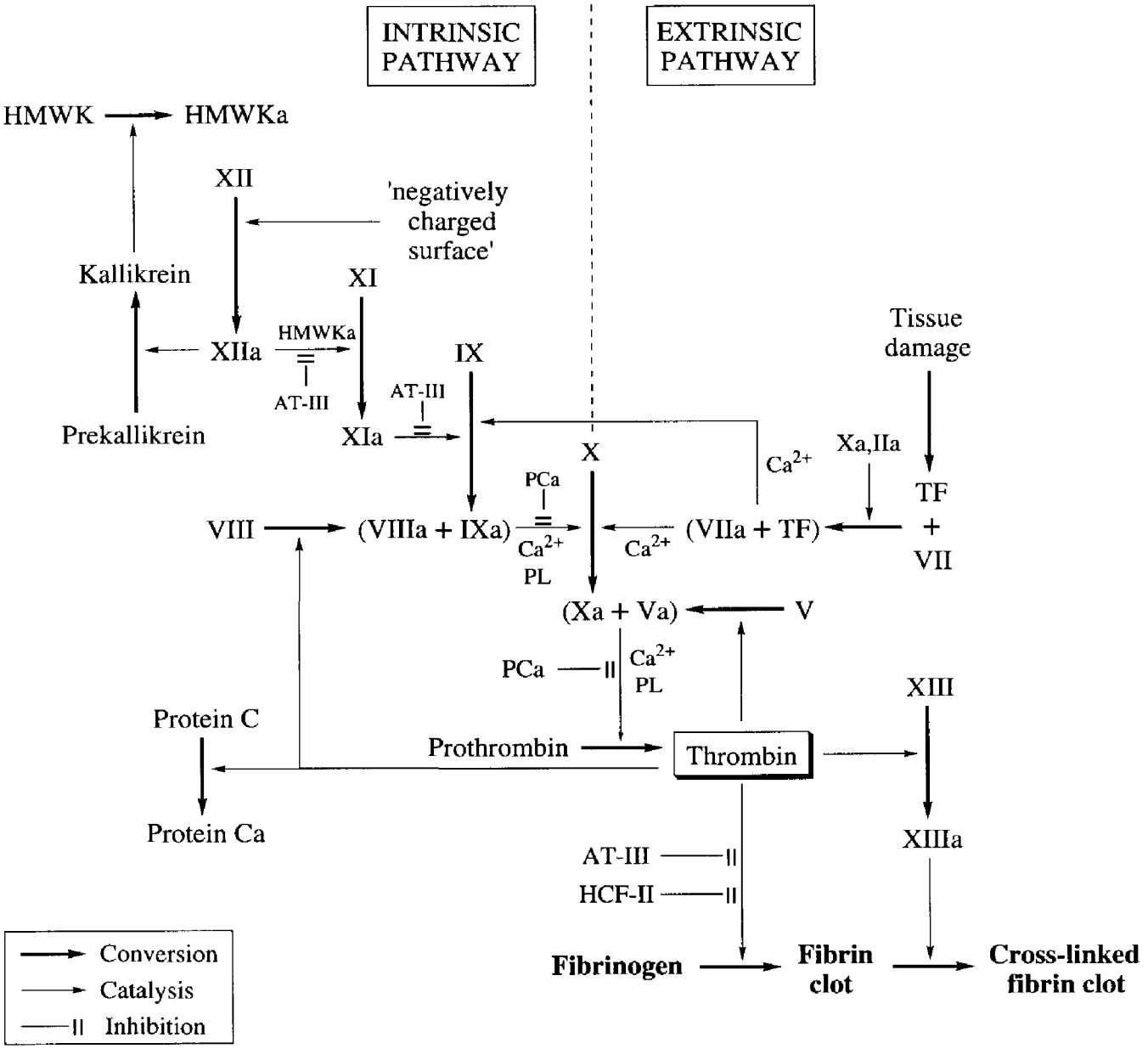
During blood coagulation a controlled sequence of proteolytic reactions, in which more than 20 inactive enzymes are converted to active proteases, takes place. Scheme 1.1 illustrates the complexity of this process.[2,3]

The principle proteins of this sequence are termed *factors*, each having a Roman numeral designation, their activated states are indicated by the suffix 'a' (*e.g.* VIIa). Activation of a factor occurs by a process of limited proteolysis in which proteins operate in pairs: one acting as enzyme, the other as substrate.[4] Because each activated enzyme can activate many other enzymes in turn, once initiated, this system eventually leads to an explosive activation of prothrombin (factor II) to give thrombin (factor IIa). Upon activation, thrombin cleaves fibrinopeptides A and B from the soluble fibrinogen by hydrolysis of four arginine-glycine bonds.[5] The resulting fibrin monomers spontaneously polymerize to form a fibrin clot. This fibrin clot is subsequently cross-linked by the action of factor XIIIa to give a stabilized and insoluble fibrin clot. This series of self-amplifying enzymatic reactions is generally referred to as the coagulation or fibrin cascade.[6]

This cascade also allows an exquisite control of the coagulation process by both positive and negative feedback loops, and by specific protease inhibitors.

Two separate initiating systems exist; the extrinsic and the intrinsic pathway.[7,8] The necessary components for activation of the intrinsic system are contained within the blood, while the extrinsic system requires a tissue factor (TF = thromboplastin) which is only released from damaged tissue and macrophages. Whereas the extrinsic system is critical in the fast initiation of fibrin formation, the intrinsic system controls the growth and maintenance of the fibrin plug. The two separate systems converge at factor X.

Scheme 1.1. Schematic overview of the fibrin cascade.



HMWK: High Molecular Weight Kinogen; TF: Tissue Factor (thromboplastin); AT-III: Antithrombin-III; PCa: activated Protein C; PL: Phospholipid; HCF-II: Heparin Cofactor-II.

1.2.2. Fibrinolysis

The principal function of the fibrinolytic system is to dissolve blood clots by degrading fibrin to soluble fragments. Fibrinolysis under non-pathogenic conditions prevents uncontrolled thrombosis while hemostatic plugs are maintained. The central enzyme is plasmin, which is generated from its inactive form by action of various proteases such as tissue plasminogen activator (t-PA).[9,10]

The fibrinolytic system involves a protease cascade which is considerably shorter than the fibrin cascade, but shares the same features of proteolytic activation: positive and negative feedback loops, and specific protease inhibitors.[9]

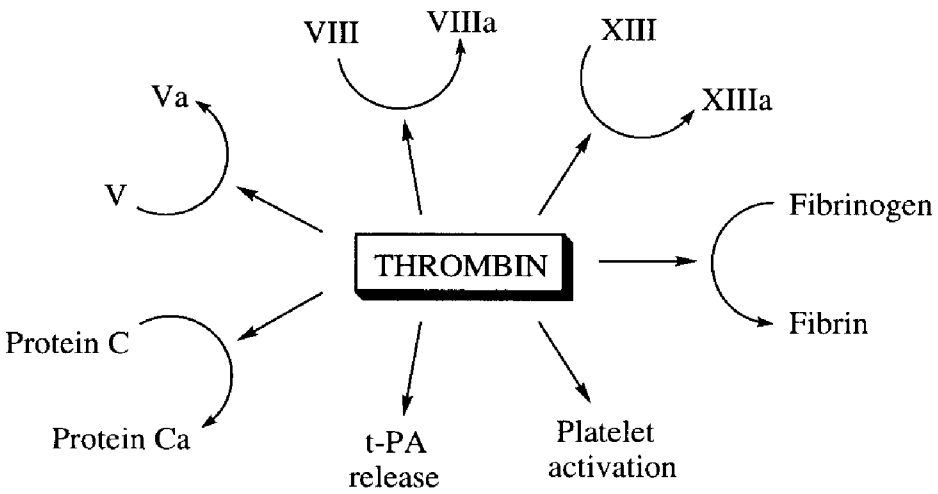
1.2.3. Thrombin

The enzyme thrombin plays a pivotal role in the maintenance of the intricate balance between coagulation and fibrinolysis.[11,12] Both free and clot-bound thrombin can convert fibrinogen to fibrin, thus promoting stabilization and propagation of the clot at a site of injury.[13] Thrombin amplifies its own production through a positive feed back loop *via* proteolytic activation of two factors in the blood cascade, factors V and VIII [Scheme 1.1]. In activating factor XIII, thrombin plays a role in stabilizing the fibrin clot. In addition, thrombin is a potent initiator of platelet activation. Since activated platelets provide a surface for the generation of thrombin which is markedly superior to plasma, this represents an additional self-amplification mechanism.[11]

Also the fibrinolysis is controlled by thrombin. In combination with thrombomodulin (an endothelial cell surface protein), it activates cofactor protein C which, upon complexation with cofactor protein S, catalyzes the inactivation of factors Va and VIIIa, and thus causes down-regulation of thrombin production.[14] Thrombin also stimulates endothelial cells to synthesize and release various antithrombotic agents such as tissue plasminogen activator (t-PA).[12] The actions of thrombin are summarized in Scheme 1.2.

In addition to its central role in coagulation and fibrinolysis, thrombin may be involved in atherosclerosis, inflammation and neurodegenerative diseases.[15]

Scheme 1.2. Actions of thrombin in coagulation and fibrinolysis.



Like most other enzymes of the fibrin and fibrinolytic cascades, thrombin is a serine protease of the trypsin family.[16] It contains a catalytic triad consisting of a serine, a histidine, and an aspartic acid residue. Although thrombin is able to cleave a variety of peptide substrates in which the amino acid sequence around the scissile bond differs considerably, it has a trypsin like specificity, *i.e.* it cleaves arginine-Aa and lysine-Aa amide bonds, with a clear preference for arginine at the P_1 position.[17] In the notation of Schechter and Berger, P_1, P_2, P_3 and P'_1, P'_2, designate substrate residues located at the amino- and carboxyl-terminal, respectively, whereas S_1, S_2, S_3, and S'_1, S'_2 indicate the corresponding binding sites of the cognate protease.[18] The P_2 position of typical protein substrates is frequently occupied by an aliphatic, hydrophobic amino acid.[19] Thrombin is particularly active towards peptidic substrates and inhibitors with a proline residue at this position.[20]

The catalytic amide bond cleavage by a serine protease can be outlined by the following, five-step mechanism [Scheme 1.3]:[21]

① formation of a non-covalent Michaelis-Menten complex between the enzyme and the substrate;

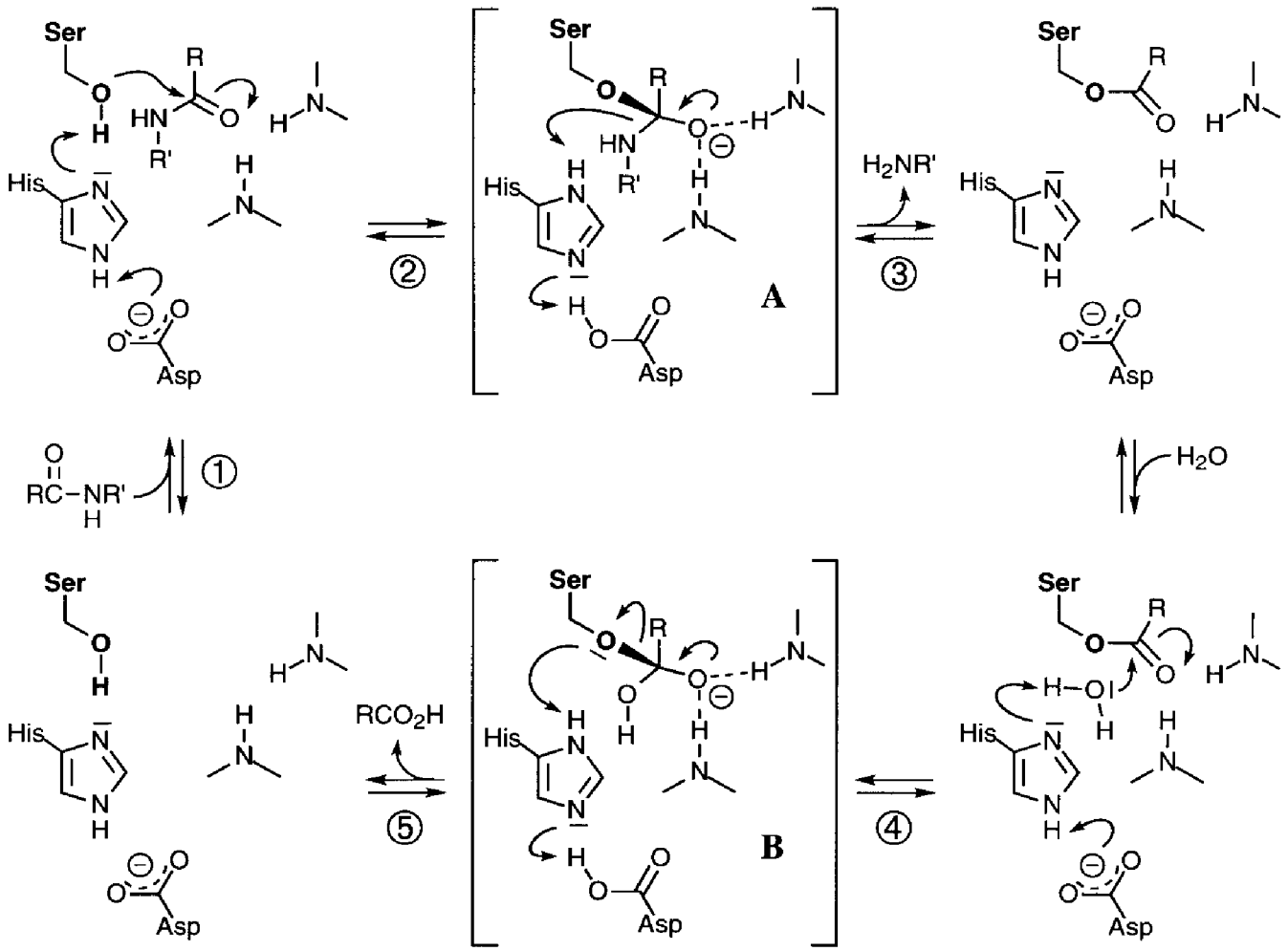
② nucleophilic attack of the hydroxyl group of the serine residue of the catalytic triad, facilitated by a proton transfer to a histidine-aspartic acid tandem, resulting in an anionic tetrahedral intermediate **A**, which is stabilized through hydrogen bonding to two ideally placed amide protons (a structure commonly referred to as 'oxanion hole');

③ collapse of this intermediate to release a peptide fragment (H_2NR') which results in *O*-acylation of the enzyme;

④ nucleophilic attack on the acylated enzyme by a water molecule, again facilitated by the histidine-aspartic acid tandem to give a second tetrahedral intermediate **B**, and

⑤ collapse of this intermediate to liberate a second peptide fragment (RCO_2H) and to set the stage for a new catalytic cycle by also liberating the enzyme.

Scheme 1.3. Schematic representation of amide bond hydrolysis catalyzed by serine proteases.



In contrast to most other serine proteases, the specificity of thrombin towards natural substrates is not solely determined by binding sites surrounding the active site. For efficient cleavage of *e.g.* fibrinogen,

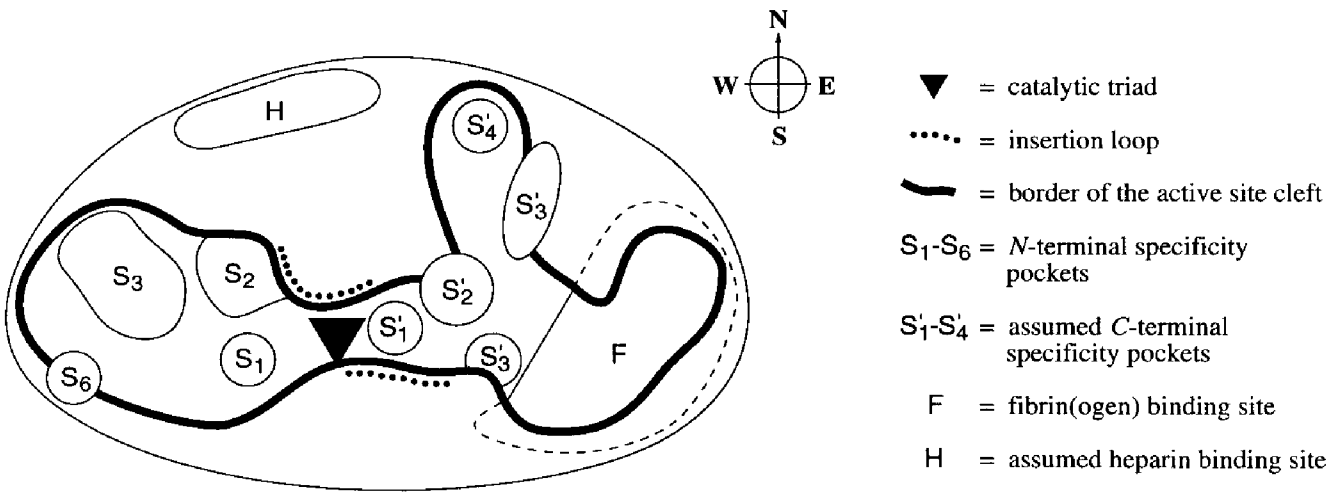
an exo site quite distant from the active site plays an important role.[22] Based upon substrate binding and sequence homology with (chymo)trypsin, models which gave a general impression of the three-dimensional structure of thrombin were proposed.[22] Fortunately, during the last few years several X-ray structures of thrombin-inhibitor complexes have been published,[22,23] thereby revealing the true 3-D structure of thrombin. The characteristics of the 3-D structure of thrombin are discussed below,[19] the X-ray data of two thrombin–inhibitor complexes are discussed in Section 1.3.2 and Section 1.4.

The most remarkable feature of the thrombin surface is the deep narrow cleft containing the catalytic triad and the adjacent substrate binding sites [Figure 1.1].[19,22] The active site cleft with its catalytically active amino acid residues extends from west to east to bind substrates from the *N*- to the *C*-terminus. The catalytic triad (Ser-195)–(His-57)–(Asp-102) does not differ from other trypsin-like serine proteases. A series of more elongated and exposed loops, especially around the active site cleft, lead to deepening and narrowing of the structure of the active site compared to that of related serine proteases. Towards the *north rim* the cleft is bordered by (Leu-59)→(Asn-62) (the so-called 60-insertion loop); loop segment (Tyr-60A)–(Pro-60B)–(Pro-60C)–(Trp-60D)–(Asp-60E) extends strikingly from the body of the enzyme and functions like a lid for the active site. The phenolic side chain of Tyr-60A covers the S_2 pocket, and Trp-60D is almost fully exposed to the solvent. Although more insertion loops which restrict access to the cleft have been identified on the south rim, *e.g.* (Leu-144)–(Gly-150), the 60-insertion loop is the most prominent and its internal conformation is generally maintained in each thrombin crystal structure known so far.[22,23] It is believed that this restricted access to the active site cleft accounts for the observed high selectivity of thrombin for certain macromolecular substrates.

The S_1 pocket has an aspartic acid residue (Asp-189) at the bottom which is capable to form strong hydrogen-bonded ion pairs with the positively charged side chain of basic P_1 residues. The hydrophobic S_2 pocket is suitable for accommodation of non-polar residues of intermediate size (Pro, Ile, Ala and Val). A second, more extended hydrophobic domain (S_3) is able to accommodate large (aromatic) hydrophobic residues (*vide infra*), and is also designated the aryl binding site.

In Figure 1.1 several more established and assumed binding sites are depicted. For an elaborate discussion on the 3-D structure of thrombin the reader is referred to the monographs of Bode *et al.*.[19,22,23]

Figure 1.1. Schematic representation of relevant thrombin features.[19]



1.3. Thrombin Inhibitors

As mentioned in Section 1.2, the processes of coagulation and fibrinolysis constitute a dynamic balance between the formation and removal of the fibrin clot. If the balance of these processes is disturbed, either excessive bleeding or thrombosis may occur. Although bleeding disorders, in particularly hemophilia, have historically been given a lot of attention, the problem of thrombosis is a much more common problem. Thrombosis causes or contributes to a variety of diseases and pathogenic conditions (*vide supra*).

For treatment or prevention of thrombosis, inhibition of coagulation or potentiation of fibrinolysis will have comparable results. Historically, inhibition of enzymes has attracted much more attention, because is was believed that potentiation of an enzymatic process was more difficult. However, potentiation of an enzyme cascade might be possible by eliminating or preventing negative regulatory feedback or inhibitory processes. Since endogenous inhibitors constitute an important negative regulatory mechanism of enzyme cascades, modulation of an inhibitor-enzyme relationship may provide such a potentiating effect. Nevertheless, in the treatment of thrombosis, pharmacodynamic intervention in the coagulation cascade is still the most popular target.

Anticoagulants can be divided into two classes: indirect thrombin inhibitors which inhibit formation of fibrin by limiting the formation of thrombin [Section 1.3.1], and direct thrombin inhibitors which involve a direct interaction of this enzyme and an inhibitor [Section 1.3.2].

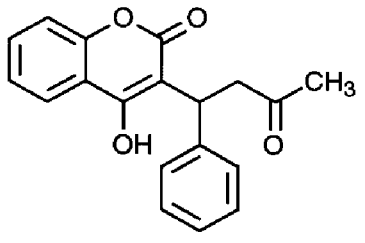
1.3.1. Indirect thrombin inhibitors

Warfarin (*i*) [Figure 1.2], a coumarin derivative, is the most commonly used, orally active anticoagulant.[24,25] The coumarin anticoagulants interrupt the fibrin cascade by inhibiting hepatic synthesis of factors II, VII, IX, X [Scheme 1.1].

Coumarins are mechanism-based, irreversible inhibitors of the hepatic microsomal $NAD(P)^+$ reductase which converts vitamin K epoxide to vitamin K.[26] This vitamin participates in the γ-carboxylation of glutamic acid in the above mentioned factors (so-called vitamin K dependent factors). The modified glutamate residues of these factors are essential for calcium binding. Without calcium, binding of the factors at the phospholipid surface (essential for their functioning [Scheme 1.1]) is hampered and the proteins circulate as non-functional precursors.[27,28]

This class of orally active compounds has a long duration but a slow onset of action (12-24 h), since they have no effect on the existing coagulation factors. Therefore, patients require careful monitoring to assure a safe level of anticoagulation. Complications of warfarin therapy (*e.g.* severe bleeding) are not uncommon.[13,25]

Figure 1.2. Warfarin, an orally active anticoagulant.



i

Heparin is the most widely used injectable anticoagulant for the treatment and prophylaxis of thrombosis.[25,29] Heparin is a heterogeneous mixture of highly sulphated glycosaminoglycan polymers with molecular weights ranging from 3000 to 100 000 Da. It is commercially derived from beef lung or porcine intestinal mucosa, and has little, if any, effect on the fibrin cascade by itself. Its mode of action is mediated primarily by antithrombin III (AT-III) [Scheme 1.1], an endogenous protein produced in the liver, that binds to and neutralizes mainly thrombin and factor Xa; however, factors IXa, XIa, XIIa and kallikrein may also be inhibited [Scheme 1.1].[30] The interaction between AT-III and these factors is dramatically enhanced by the presence of heparin which binds to and causes a conformational change in AT-III.

In addition to its association with AT-III, heparin forms a complex with a second endogenous thrombin-inhibiting protein, heparin cofactor II (HCF-II).[31] Heparins also promote fibrinolysis by stimulating release of tissue plasminogen activator. This effect may contribute to its antithrombotic activity.[32]

Unlike warfarin (***i***), heparin has a rapid onset of action, but must be administered parenterally.[25] Like warfarin, it can cause bleeding disorders, and because of its mechanism of action, heparin is ineffective in patients having AT-III deficiency.[33] Furthermore, as AT-III is consumed in the process of coagulation, low AT-III plasma concentrations may also cause low response to treatment with heparin. This is likely to occur in situations of extensive thrombosis.[25]

Low molecular weight heparin may offer some advantages over (normal) heparin with respect to side effects. Most low molecular weight heparins are still large molecules, but synthetic derivatives as small as pentasaccharides are able to preserve the antithrombotic activity.[34] Considerable effort is directed towards the development of highly active, synthetic oligosaccharides for the treatment of thrombosis.[35]

1.3.2. Direct thrombin inhibitors

Clinical experience with indirect thrombin inhibitors provides the basis for the belief that a direct thrombin inhibitor may offer a safe and efficacious treatment of thrombosis. A variety of compounds, both natural and synthetic, have been found to inhibit the fibrinogenolytic activity of thrombin.

Hirudin, a naturally occurring acidic 65-amino acid polypeptide isolated from medicinal leeches, is the most potent and selective thrombin inhibitor known.[36] The tight binding of hirudin to thrombin (K_i = 0.3 pM) is achieved through interactions at both the hydrophobic binding site (S_2 pocket) adjacent to the active site, and to the remote fibrin(ogen) binding site (F) [Figure 1.1].[37] Hirudin is highly selective in its binding to thrombin and has no inhibitory activity against other related enzymes. The superior therapeutic profile of the direct-acting hirudin compared with the indirect-acting heparin in animal models of thrombosis have made it an attractive target for drug development.[37] However, hirudin and analogues (so-called hirulogs) are high molecular weight polypeptides that suffer from a short duration of action and must be administered parenterally.

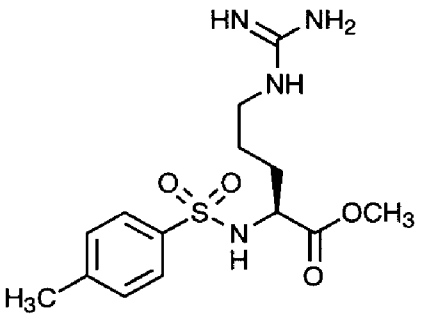
The development of synthetic, direct thrombin inhibitors can be traced back to TAME (***ii***), *i.e.* N^{α}-tosyl-L-arginine methyl ester [Figure 1.3].[38,39] The closely related analogue ***iii*** was the first competitive inhibitor of thrombin.[39] A vast amount of research has been spent on analogues of TAME: as much as 560 compounds have been published. These inhibitors are commonly referred to as active-site-directed thrombin inhibitors as they interact with the S_1 binding pocket. All of these compounds can be characterized by three subunits: (1) an arginine residue, sometimes with a modified guanidino

group *e.g.* a benzamidine; (2) an aromatic group at the N^{α} position of arginine, and (3) a hydrophobic substituent at the arginine carboxyl group.

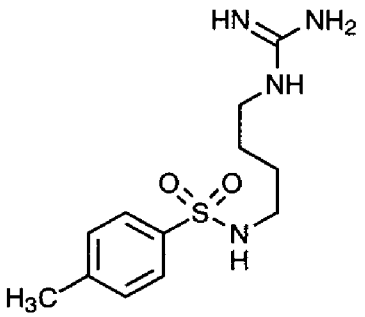
Very potent, extensively studied representatives of this class of active-site-directed inhibitors are argatroban (***iv***) and NAPAP (***v***) with K_i-values of 19 and 6 nM respectively.[40,41] These compounds are very specific for thrombin and their toxicity is low. However, oral activity is limited because of the low adsorption of these compounds. Argatroban (***iv***) is one of the most selective thrombin inhibitors known, and is marketed in Japan as an intravenous agent.[13]

X-ray crystal studies of argatroban and NAPAP bound to thrombin have shown that these compounds bind in a compact, U-type conformation (referred to as *inhibitor-like* binding) different from the peptidic substrates and peptide derived inhibitors (*vide infra*).[22] This structural information was utilized recently in designing the potent and selective agent ***vi*** (K_i = 0.27 nM) which is 7000-fold less effective towards trypsin.[42]

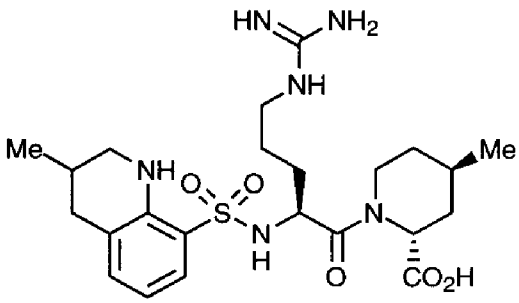
Figure 1.3. Arginine derived, active-site-directed thrombin inhibitors.



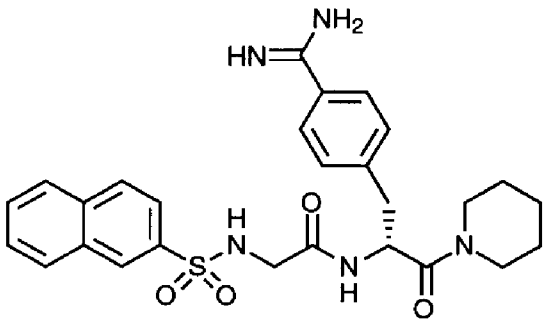
ii ($K_i = 1 \times 10^6$ nM)



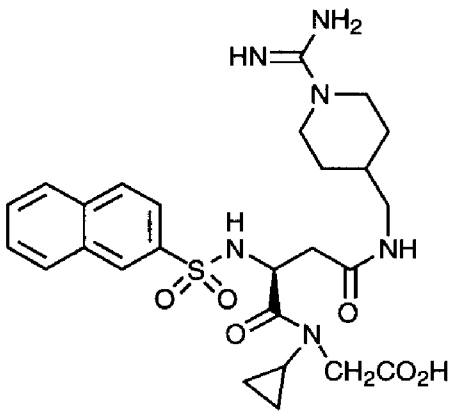
iii ($K_i = 13 \times 10^6$ nM)



iv (K_i = 19 nM)



v (K_i = 6 nM)



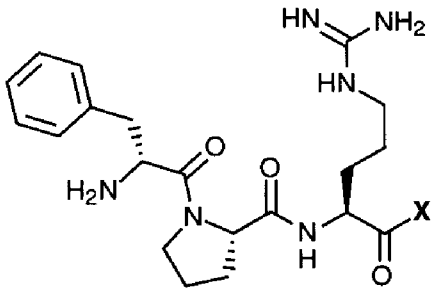
vi (K_i = 0.27 nM)

A second class of active-site-directed thrombin inhibitors is based upon the structure of thrombin's natural substrates. In these natural substrates, besides the presence of an arginine residue at the *C*-terminus of the scissile bond (the P_1 residue), the frequency of a proline residue at the P_2 position is noteworthy. In contrast, the variety of amino acids in the P_3 position is large. In an effort to find an inhibitor with an optimal tripeptide sequence, Bajusz *et al.* found that a D-phenylalanine residue at the

P_3 position resulted in the highest affinity.[43,44] It is now believed that the Phe-8 of the fibrinogen A α-chain binds to the enzyme in a similar way as D-Phe in the tripeptide sequence.[22]
The D-Phe–Pro–Arg motif [Figure 1.4] is found in a large series of peptide-derived thrombin inhibitors. Introduction of electrophilic groups at the *C*-terminus of the tripeptide, to establish an additional interaction with the active site, resulted in a number of potent, irreversible and reversible thrombin inhibitors.[22]

In PPACK (***vii***), an irreversible thrombin inhibitor, the *C*-terminal carboxyl group is replaced by a chloromethyl ketone.[45] As the solid-state structure of the inhibitor-enzyme complex shows, PPACK inactivates thrombin by irreversible alkylation of the active site His-57.[22,46] Furthermore, the carbonyl group forms a hemiketal with the active site Ser-195 hydroxyl group, in which the negatively charged oxygen atom is stabilized, through hydrogen bonding by the oxanion hole (comparable to intermediate **A** in Scheme 1.3). The P_1, P_2, P_3 side chains of PPACK occupy the S_1, S_2, S_3 pockets, respectively (*substrate-like* binding).
Arginine peptide aldehydes and ketones, *e.g.* ***viii*** and ***ix***, also possess high antithrombin activity.[13] The mechanism of action of these reversible, tight-binding inhibitors is also likely to involve a tetrahedral adduct. By introducing an electron-withdrawing group (*e.g.* a trifluoromethyl group as in ***ix***) the carbonyl group becomes even more susceptible to hemiketal formation, which results in a more potent inhibitor.

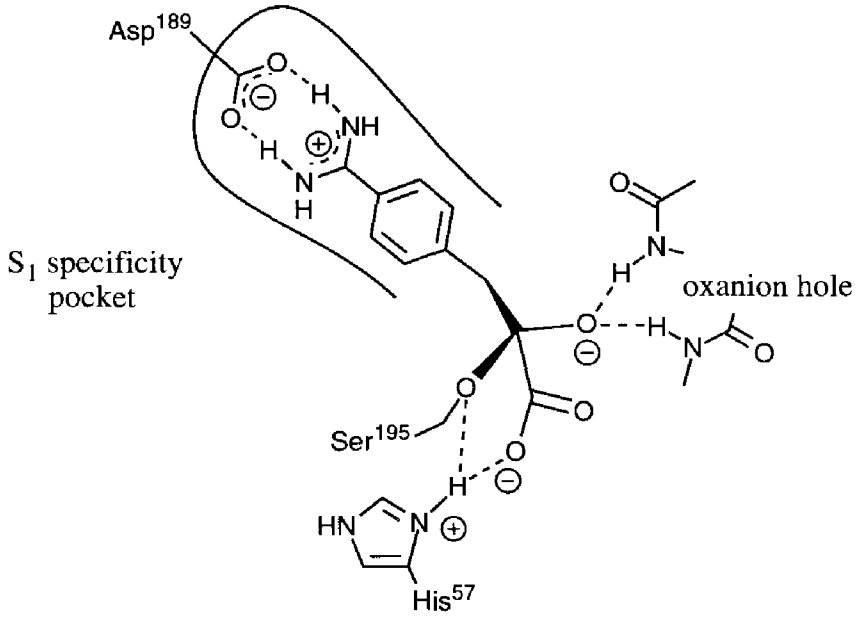
Figure 1.4. Electrophilic tripeptide thrombin inhibitors.



vii : **X** = CH_2Cl (PPACK)
viii: **X** = H (K_i = 7.5 nM)
ix : **X** = CF_3 (K_i = < 1 nM)

Also the α-keto carboxylic acid derivative 4-amidinophenylpyruvic acid (APPA) [Figure 1.5], containing an electrophilic carbonyl group is a potent inhibitor of both trypsin and thrombin (K_i-values of 1.6 and 6.5 μM, respectively).[47] The X-ray structure of the APPA-trypsin complex [Figure 1.5] showed the expected interactions. The amidinophenyl group of APPA resides in the S_1 pocket of trypsin and, comparable to PPACK, also an interaction of the hydroxyl group of Ser-195 with the carbonyl group of the pyruvate unit was observed. The hemiketal structure is stabilized by hydrogen bonding to the oxanion hole.[48]
Because these hemiketal enzyme-inhibitor complexes mimic the postulated tetrahedral intermediate **A** during substrate proteolysis [Scheme 1.3], the electrophilic keto-derivatives are often referred to as transition-state analogues to provide an attractive rationale for the increased affinity of this type of electrophilic inhibitors.[49,50] However, transition-states are characterized by incomplete bonds and awkward geometries and no compound is likely to resemble in detail this least stable of structures on the pathway from substrate to product. Therefore, we prefer to term these inhibitors just *mechanism-based* inhibitors rather than transition-state analogues.

Figure 1.5. Schematic representation of the APPA-trypsin active site interactions.



Many different electrophilic tripeptide inhibitors have been, and are being, developed. The challenge to meet is to design a highly potent and selective, orally active compound. However, compared to the simple arginine-derived direct thrombin inhibitors such as argatroban (*iv*) and *vi* [Figure 1.3], in general, the tripeptide derived inhibitors prepared so far are less selective with respect towards trypsin and plasmin inhibition.[51]

1.4. Cyclotheonamide, a Potent Thrombin Inhibitor of Natural Origin

1.4.1. Structure of Cyclotheonamide A/B

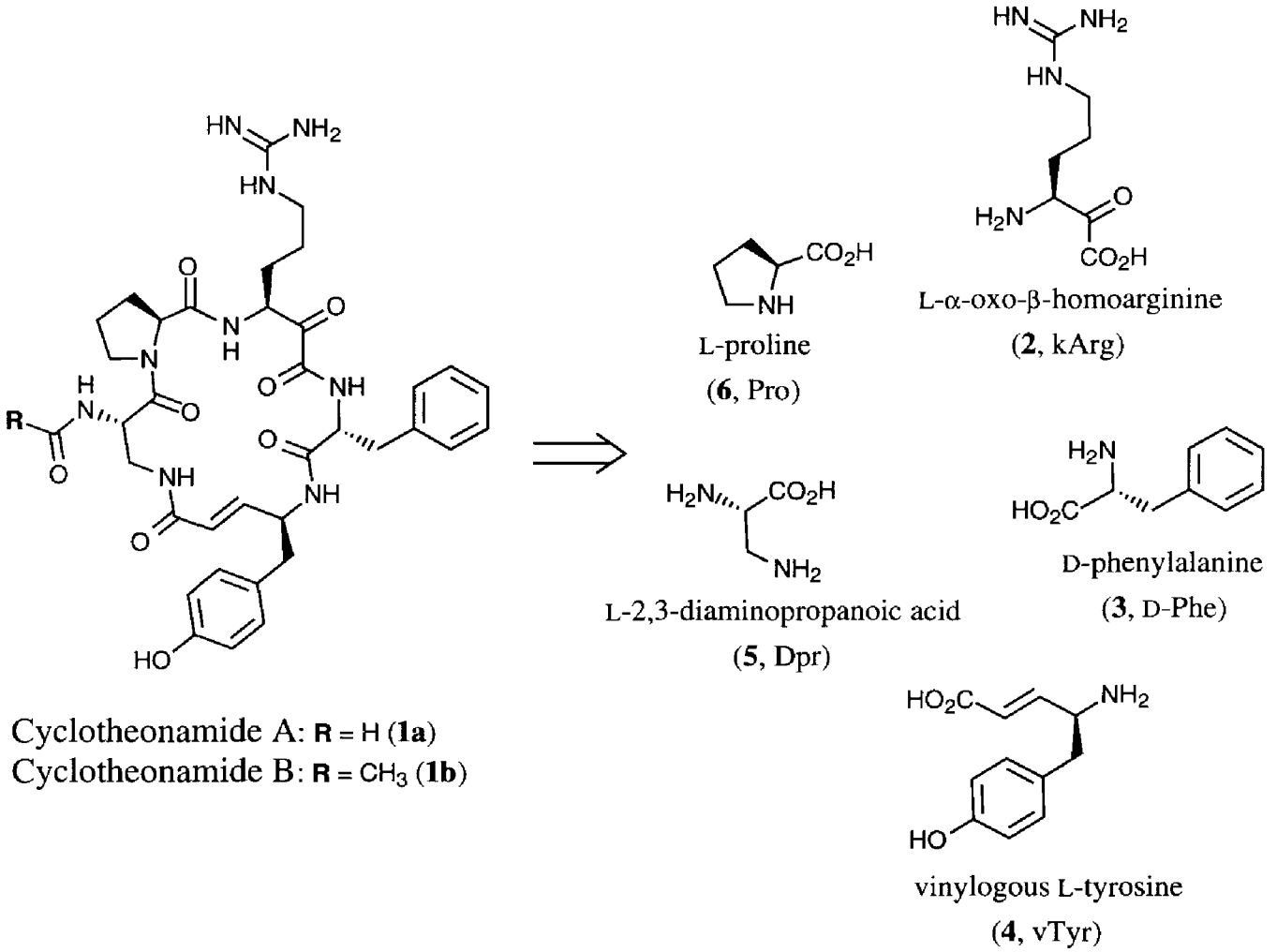
In 1990, two potent thrombin inhibitors, Cyclotheonamide A (**1a**) and B (**1b**) [Scheme 1.5], featuring an arginine moiety with an additional electrophilic α-keto amide group were reported.[52] These cyclic compounds, isolated in low yield from the marine sponge *Theonella swinhoei*, consist of five amino acid residues. Besides the proteinogenic amino acid L-proline (**6**, Pro), the unnatural enantiomer of a proteinogenic amino acid, D-phenylalanine (**3**, D-Phe), and three uncommon, non-proteinogenic amino acids, *i.e.* a N^{α}-acylated 2,3-diaminopropanoic acid (**5**, Dpr) and the hitherto unknown α-oxo-β-homo-arginine (**2**, kArg) and a vinylogous tyrosine (**4**, vTyr), are present.

The combination and cyclic arrangement of these five amino acids are of particular interest for the following reasons. Normally, even small linear peptides can adopt different low-energy conformations, which may exhibit different biological activities. The ring structure of **1** reduces dramatically the number of possible conformations and thus might have a beneficial effect on the compound's selectivity. The cyclic structure might also improve the poor farmacokinetic properties usually observed for peptide-derived structures, as the lack of polar end-groups improves the absorption from

the gastrointestinal tract (bioavailability), and also enhances the resistance against degradation by peptidases (biostability). Moreover, the presence of a D-amino acid and of three non-proteinogenic amino acids in Cyclotheonamide is expected to have an additional beneficial effect on the biostability.

On the basis of the above described structural features one might argue that Cyclotheonamide is a true peptide-mimic rather than a peptide.[53]

Scheme 1.5. Cyclotheonamide and its constituent amino acids.



At the onset of our research on Cyclotheonamide, we anticipated, on the basis of molecular modelling studies of the Cyclotheonamide-thrombin complex [Chapter 2.3], that the mechanism of thrombin inhibition was likely to exhibit similarities to the APPA-mechanism. In other words, the α-keto amide unit might be involved in the formation of a stabilized hemiketal, an idea that was shared by other researchers.[54]

1.4.2. Solid-state structures of Cyclotheonamide-serine protease complexes

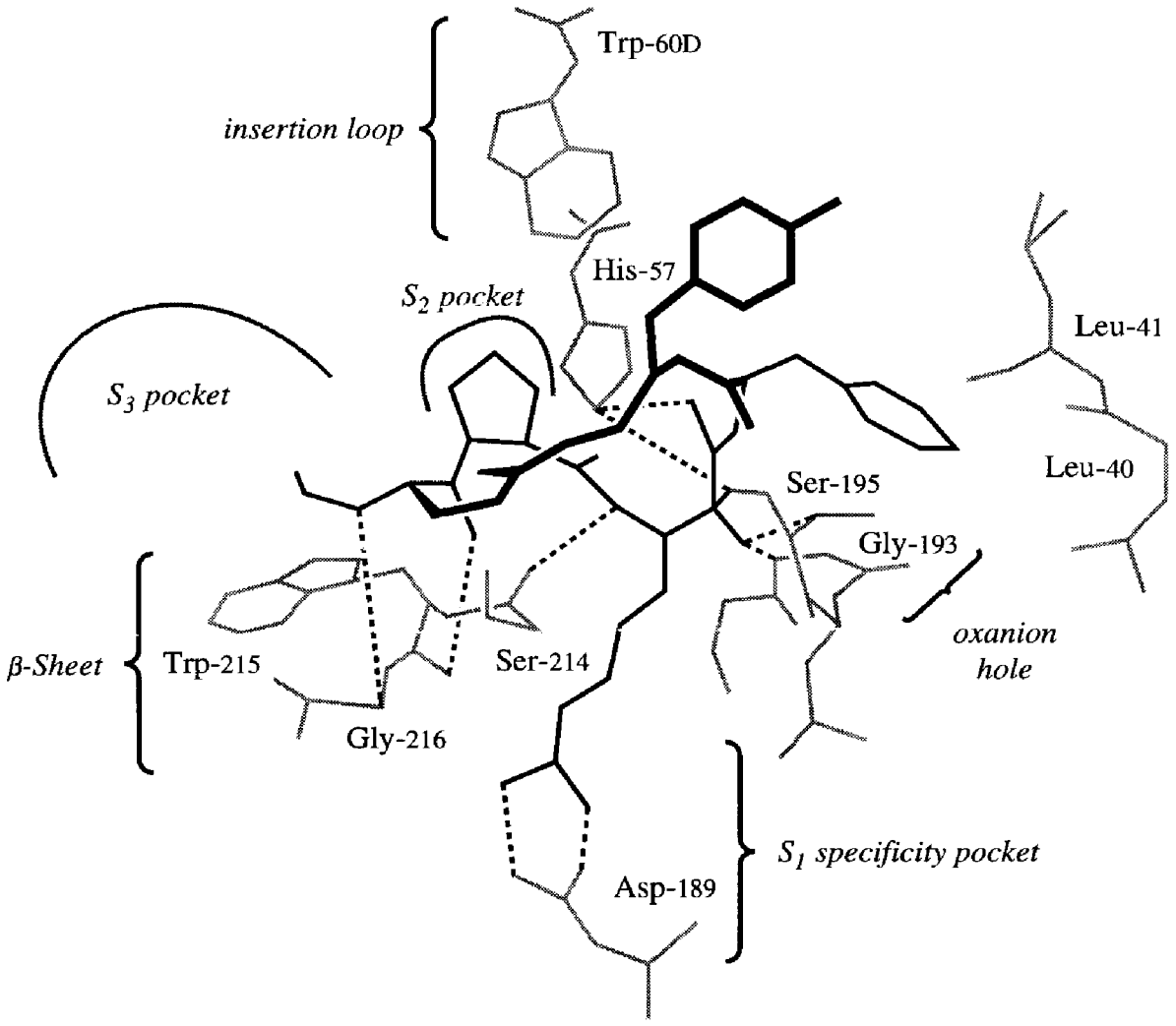
The assumed mechanism-based mode of action was confirmed in 1993 by an X-ray study by Maryanoff *et al.* of the Cyclotheonamide A–human-α-thrombin–hirugen complex.[55-57]

The solid-state structure shows the following characteristic features [Scheme 1.6]:[58]

- The hydroxyl group of Ser-195 of the catalytic triad of thrombin interacts with the electrophilic keto group of the kArg unit of Cyclotheonamide, to form a hemiketal structure. The tetrahedral adduct is stabilized by an intricate hydrogen-bonding network, in which the anionic oxygen interacts with amide hydrogen atoms of Gly-193 and Ser-195 (oxanion hole), the γ-oxygen of Ser-195 is also hydrogen-bonded to His-57.
- The Pro-Arg motif interacts in the usual way with thrombin. The arginine side chain occupies the S_1 pocket in which the guanidinium group participates in a double-bonded ion pair with Asp-189, while the proline ring resides in the hydrophobic S_2 pocket. Furthermore, the -NH-C-C(O)-Pro-Arg unit forms a hydrogen-bonded double-stranded antiparallel β-sheet with (Ser-214)–(Trp-215)–(Gly-216).
- The D-Phe unit occupies a hydrophobic domain and interacts with (Leu-40)–(Leu-41).
- And, finally, the hydroxyphenyl group of the vTyr residue is believed to be involved in an aromatic stacking interaction with Trp-60D of the insertion loop.

Noteworthy is the vacant, large hydrophobic S_3 pocket, which is normally occupied by D-Phe in tripeptide inhibitors (*vide supra*).

Scheme 1.6. Schematic representation of the Cyclotheonamide A-thrombin complex.



A crystallographic study of the binary Cyclotheonamide–(bovine-trypsin) complex, also published in 1993, showed that in this complex many structural features of the ligand-enzyme interactions are comparable to those observed in the Cyclotheonamide-thrombin complex.[59,60] The main distinctions relate to the orientations of the vTyr and D-Phe aromatic groups. The interaction of the hydroxyphenyl group of the vTyr residue with the Trp-60D residue as observed in the complex with thrombin, was found to be absent; the hydroxyphenyl group is therefore exposed to the solvent. The reason is that trypsin lacks the 60-insertion loop found in thrombin. In the trypsin complex, the phenyl ring of the D-Phe residue of Cyclotheonamide occupies a hydrophobic groove formed by Tyr-39 and Phe-41, whereas in the thrombin-Cyclotheonamide complex this hydrophobic interaction is established by Leu-40 and Leu-41.
The small differences in the steric fit between the Cyclotheonamide-thrombin and Cyclotheonamide-trypsin X-ray structures can not explain the greater effectiveness of Cyclotheonamide for inhibition of trypsin (K_i = 0.2 nM) relative to thrombin (K_i = 1.0 nM).

1.4.3. Enzyme inhibition by Cyclotheonamide

Lewis *et al.* showed that Cyclotheonamide exhibits slow-tight-binding kinetics, which means that the true steady-state binding situation is only slowly reached, and therefore special kinetics are required for calculating binding constants.[61] The K_i-value for thrombin (1.0 nM), determined under slow-binding conditions, was found the be much lower than originally reported.[52,55] Furthermore, it was shown that Cyclotheonamide lacks selectivity, as it also inhibits trypsin (K_i = 0.2 nM) and tissue plasmin (K_i = 12 nM), and several other related serine proteases.

1.5. Scope of this Investigation

The intriguing structure and biological activity of Cyclotheonamide prompted us to start an investigation on this natural peptide-mimic. First of all, it was challenging to verify the postulated, mechanism-based mode of action. If the α-keto amide moiety indeed is involved in a reversible, covalent interaction with the enzyme, the hitherto unknown α-oxo-β-homoarginine residue offers the possibility to develop a novel class of low molecular weight, active-site-directed thrombin inhibitors, by flanking the electrophilic keto amide unit with a peptide motif (linear or cyclic) that spans the active site region.[62] This development might yield structures with improved selectivity compared to the electrophilic tripeptide inhibitors reported thus far.

Since Cyclotheonamide was only isolated in low yields from its natural source, a total synthesis was to be devised in order to prepare enough material to study the characteristics of the natural product. Furthermore, since Cyclotheonamide also inhibits several other serine proteases, the synthetic protocol developed for the natural product should be flexible to allow rapid and straightforward preparation of analogues to explore the issues of selectivity and potency.

While our research progressed, our expectations grew as we anticipated that the vast wealth of information that gradually came available from solid-state structures of inhibitor-enzyme complexes could be utilized for the rational design of more potent and more selective Cyclotheonamide analogues. As is apparent from the X-ray structure of the Cyclotheonamide-thrombin complex, improvement of the steric fit especially with regard to the P_3-S_3 interaction should be feasible.

These considerations formed the basis of our research, the results of which are presented in the next chapters. In Chapters Three to Five, the total synthesis of Cyclotheonamide B, based on a synthetic strategy discussed in Chapter Two, is presented. The preparation of several Cyclotheonamide analogues, and their biological data are given in Chapter Six and Chapter Seven, respectively. In Chapter Eight our approach to Cyclotheonamide and analogues is summarized and compared to the synthetic contributions of other research groups in this field.

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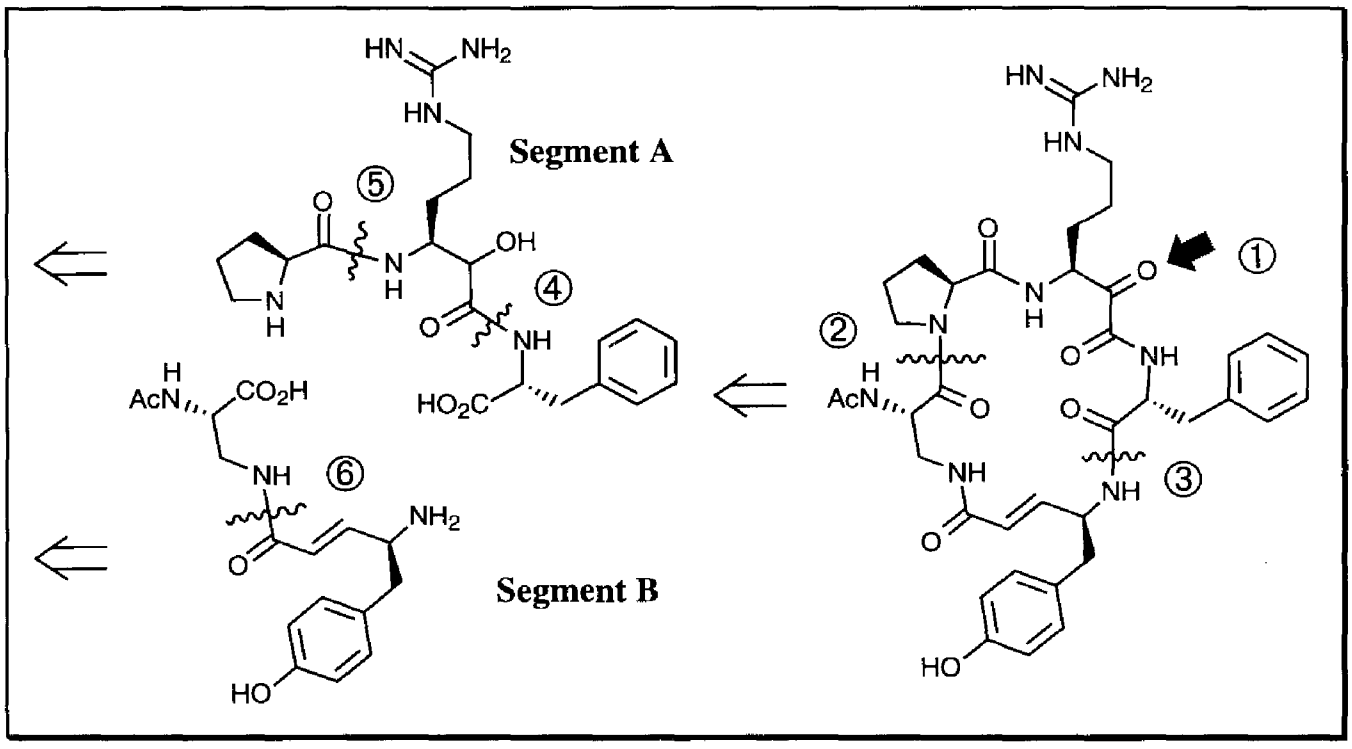
CHAPTER TWO

Synthetic Strategy



Abstract

In this chapter, our plan towards the total synthesis of Cyclotheonamide B is presented.
In order to devise a flexible and straightforward route which would also allow the preparation of analogues, a convergent approach using the two key intermediates **A** and **B** was anticipated. On the basis of a retrosynthetic analysis these key intermediates and their starting materials were defined. A synthetic route for their elaboration into the target compound was set out taking into account several considerations regarding protecting group strategy and functional group transformation.

Besides our own approach, a concise summary and comparison of approaches by other research groups is given.

2.1. Introduction

Whereas at the onset of our studies in 1991, no synthesis of Cyclotheonamide A/B had been reported, four syntheses were described,[1-4] once we had completed our total synthesis.[5]

One might wonder why a molecule isolated and characterized as late as 1990 has attracted so much attention: over 16 papers on isolation and characterization,[6-8] synthetic aspects,[1-5,9-12] enzyme inhibition,[4,13-15] X-ray data,[4,11,12,14,15] and conformational analysis[16] have appeared in the literature since. The answer to this question lies in the unique structure as well as in the biological activity of Cyclotheonamide. It is a true peptide-mimic, featuring two hitherto unknown amino acids, *viz.* α-oxo-L-β-homoarginine (**2**) and a L-tyrosine derived α,β-unsaturated γ-amino acid (**4**) [Chapter 1.4.1]. Moreover, it is a potent inhibitor of several serine proteases. Of potential therapeutic value is its inhibitory activity towards thrombin, a serine protease playing a crucial role in the bioregulation of thrombosis and hemostasis. The mode of action of Cyclotheonamide is based on the reversible interaction of a hydroxyl group of the enzyme's active site with the α-keto group of the arginine moiety [Chapter 1.4.2]. That this novel mode of action, together with the unusual structural features, forms a basis for the design of novel thrombin inhibitors was readily recognized by a wide variety of researchers, at universities and research institutes as well as at pharmaceutical companies.

As stated in their reports, the main goal of all four research groups that completed the synthesis of Cyclotheonamide A and/or B was to devise a suitable route to Cyclotheonamide and analogues in order to develop new thrombin inhibitors. In this chapter a summary of these four syntheses is presented [Section 2.2]. A comparison of the different approaches shows some remarkable resemblances as well as differences in the synthetic strategies, in the preparation of the unusual amino acids and in the protecting group strategy used.

In Section 2.3 our synthetic strategy towards Cyclotheonamide B and its analogues is given. Although this strategy turned out to have some features in common with the four approaches published, especially with that of Maryanoff *et al.*,[11] we feel it deserves a place of its own, in particular with regard to protecting group strategy used.

2.2. Summary and Comparison of Cyclotheonamide Syntheses

As stated above, no (partial) synthesis of Cyclotheonamide A/B had been published at the start of our studies. At this moment five total syntheses have been published by different groups, including ours.[1-5] Here follow, in the order of appearance, some features and relevant details of the achievements of the four other research groups.

The first synthesis, reported by Hagihara and Schreiber,[2] employs a linear approach, whereas Wipf and Kim,[3] Maryanoff *et al.*,[4] and Shiori *et al.*,[5] use a convergent [3 + 2] fragment-condensation approach, albeit with different key intermediates and different protecting group strategies. The overall

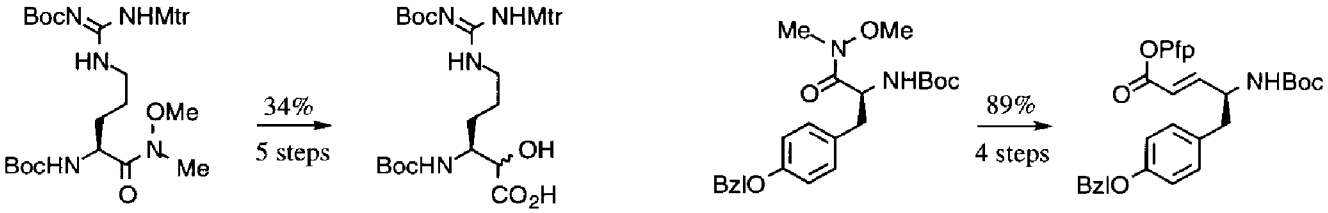
yields reported by the first three groups range from 0.8 to 1.8%; the Shiori group reported an overall yield of 12% (*vide infra*). Strikingly similar in all four approaches is the construction of the α-keto amide unit, *viz.* by oxidation of an α-hydroxy-β-homoarginine unit *prior* to final deprotection. The syntheses of the hitherto unknown amino acids, *i.e.* α-hydroxy-β-homoarginine (hArg) and vinylogous tyrosine (vTyr), are very much the same in all four syntheses. Both amino acids are prepared from the corresponding α-amino acids, using α-amino aldehydes as intermediates. The use of an elaborate set of protective groups, especially for arginine and derivatives, proved to be the key to ultimate success. It is remarkable that in all four syntheses an arylsulfonyl protecting group, *viz. p*-toluenesulfonyl (Ts) or 2,5,6-trimethyl-4-methoxybenzenesulfonyl (Mtr), is used to block the guanidino group of the arginine derivatives.

Schemes 2.1 and 2.2 summarize the linear approach of Schreiber's group at Harvard University.[2] For the homologation of $N^{\alpha,\omega'}$-(bis-Boc), N^{ω}-Mtr-arginine *N,O*-dimethylhydroxamate (**7**) to α-hydroxy-β-homoarginine derivative **8**, Schreiber used a method developed originally by Seebach, employing tris(methylthio)methyllithium as a carboxylate anion equivalent. As part of a study on the properties of vinylogous peptides, a general route to vinylogous amino acids had already been devised by Schreiber.[17] This approach was applied to protected tyrosine **9** to give vinylogous tyrosine derivative **10**.

The linear pentapeptide **12** was obtained by four consecutive amide couplings in the C→N direction, starting with proline methyl ester (**11**). Macrolactamization of **12** involving the β-amino group of the homoarginine unit as nucleophile was achieved by the following four-step process. Removal of the phenacyl (Pac) group followed by pentafluorophenyl (Pfp) ester formation at the *C*-terminus, and selective removal of the *N*-terminal Boc group, afforded after neutralization and treatment with 4-(dimethylamino)pyridine, cyclopentapeptide **13** in 31% yield. Following oxidation of the hydroxyl group (which was left unprotected during the entire synthesis) the complete regiment of protecting groups was removed in a single step to give Cyclotheonamide B in an overall yield of 0.8%, starting from arginine derivative **7**.

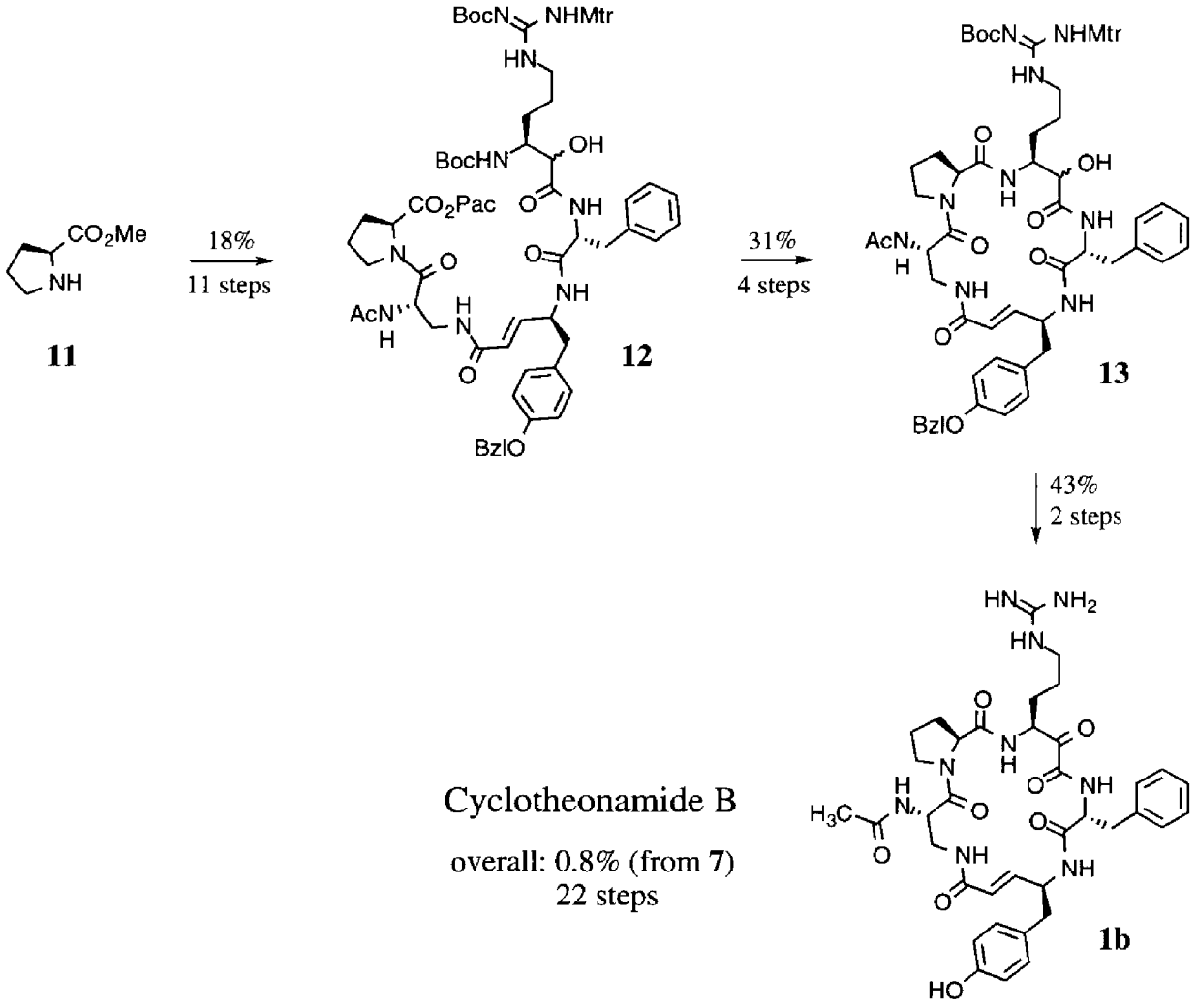
In order to elucidate the stereochemistry at the homoarginine residue of Cyclotheonamide (not defined in Fusetani's original paper) both the epimers with *R*-and *S*-kArg were prepared separately using the reaction sequences shown in Scheme 2.1 and 2.2. Based upon the potent thrombin inhibition by the 3*S* epimer, the α-oxo-β-homoarginine unit was assigned the *S*-configuration Furthermore, the configuration of the vinylogous tyrosine unit (originally assigned *R* by Fusetani) was corrected to *S*, by comparison of the NMR-data of the two synthetic epimers with a sample of the authentic natural product.

Scheme 2.1. Synthesis of β-homoarginine **8** and vinylogous tyrosine **10** according to Schreiber *et al.*.[2]



7 **8** **9** **10**

Scheme 2.2. Synthesis of Cyclotheonamide B according to Schreiber *et al.*.[2]

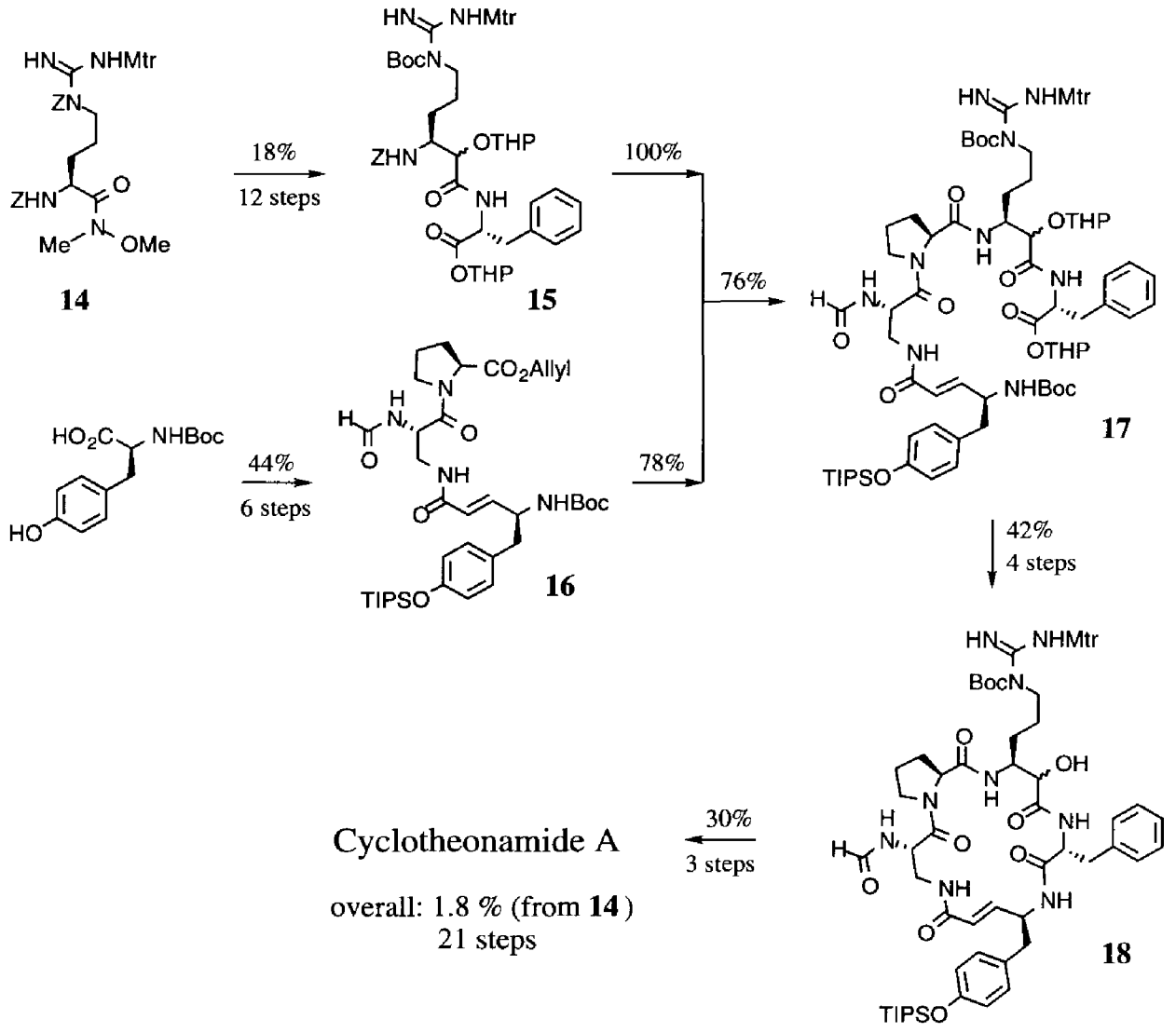


Ac: acetyl; Boc: *t*-butyloxycarbonyl; Bzl: benzyl; Pac: phenacyl;
Mtr: 2,5,6-trimethyl-4-methoxybenzenesulfonyl; Pfp: pentafluorophenyl.

Wipf and Kim (University of Pittsburgh) devised a convergent approach in which the macrocycle was constructed by condensation of a tripeptide (**15**) and a dipeptide (**16**) [Scheme 2.3].[3]
For the preparation of the α-hydroxy-β-homoarginine derivative from an arginine derivative (**14**) with a double protected guanidino group the cyanohydrin synthesis was applied. The N^{δ}-Z group was found to be very base-sensitive, and was replaced *en route* with the less base-sensitive Boc group. The hydroxyl group of the β-homoarginine unit was protected as a tetrahydropyranyl ether (see **15**).

Their final approach, using dipeptide **15** and tripeptide **16** as key intermediates, is depicted in Scheme 2.3. In an earlier report, in which the synthesis of the α-hydroxy-β-homoarginine derivative was disclosed, a different [3 + 2] strategy was presented.[10]
The four-step macrocyclization of **17**, *via* the pentafluorophenyl ester of the D-phenylalanine unit, gives cyclopentapeptide **18** in 42% yield. Oxidation of the hydroxyl group and the final two-step deprotection gives Cyclotheonamide A in 1.8% overall yield from arginine derivative **14**.

Scheme 2.3. Synthesis of Cyclotheonamide A according to Wipf and Kim.[3]



Boc: *t*-butyloxycarbonyl; Mtr: 2,5,6-trimethyl-4-methoxybenzenesulfonyl; THP: tetrahydropyranyl; TIPS: triisopropylsilyl; Z: benzyloxycarbonyl.

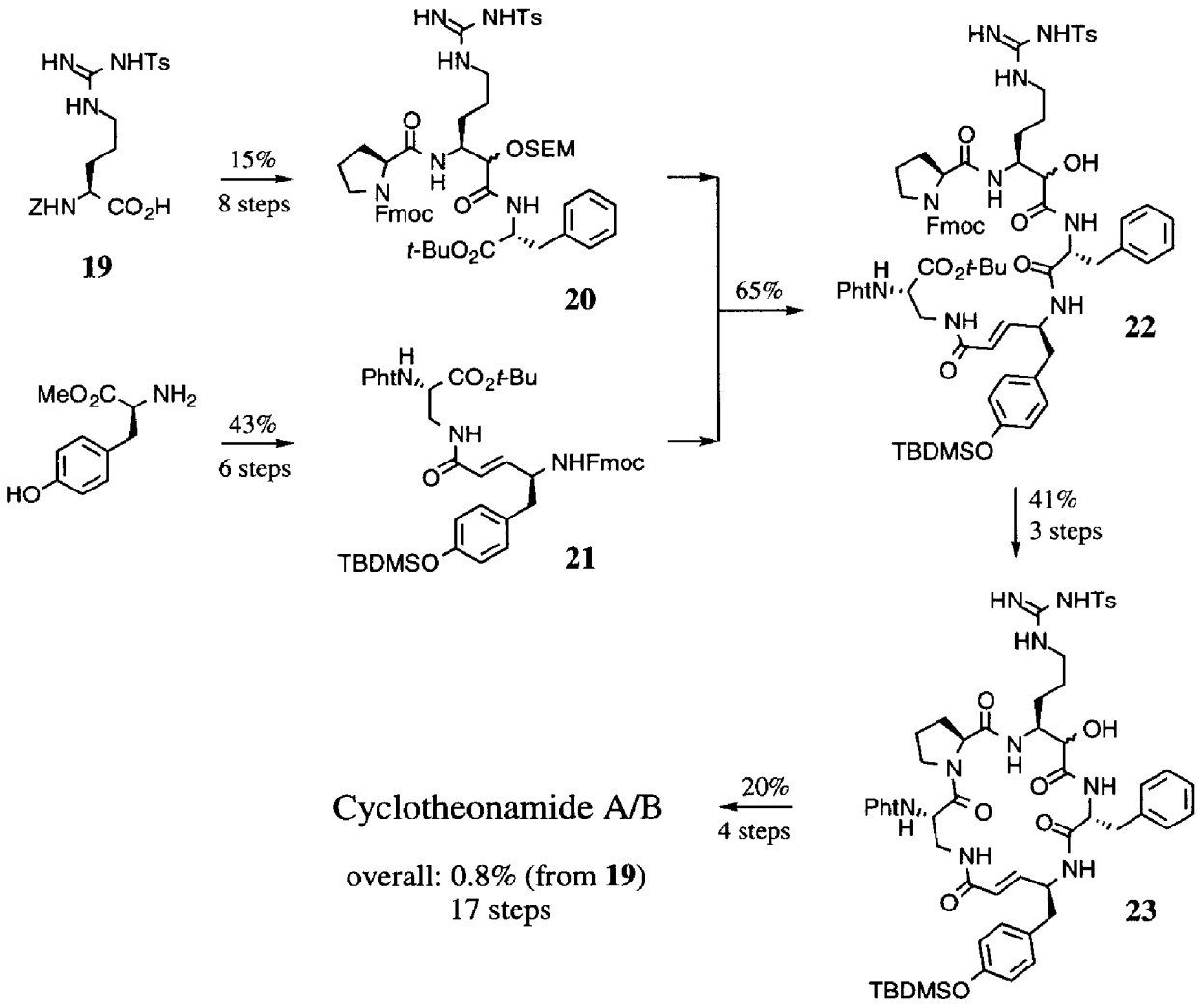
In the convergent [3 + 2] approach of the Maryanoff group (Johnson Pharmaceutical Research Institute) in co-operation with K.C. Nicolaou (The Scripps Research Institute) and A. Tulinsky (Michigan State University),[3,11,12] the β-homoarginine unit was also obtained *via* cyanohydrin formation [Scheme 2.4]. However, in contrast to Wipf and Schreiber, a mono-protected guanidino group was used throughout the entire synthesis. Temporary protection of the α-hydroxyl group in the β-homoarginine derivative was found to be necessary to prevent intramolecular reactions of this hydroxyl group. The macrocycle was constructed by condensation of tripeptide **20** and dipeptide **21**.

Macrolactamization of **22**, in a three step procedure involving cleavage of the *N*-terminal Fmoc group, deprotection of the *t*-butyl ester of the diaminopropanoic acid unit, and amide formation by using dicyclohexylcarbodiimide/1-hydroxybenzotriazole, gave protected cyclopentapeptide **23** in 41% yield. Cleavage of the phtaloyl (Pht) group at the α-amino group of the diaminopropanoic acid unit in **23** was followed by introduction of the formyl or acetyl group. Subsequent oxidation of the hydroxyl

group and final deprotection, furnished Cyclotheonamide A/B in 0.8% yield starting from arginine derivative **19**.

The synthesis of Maryanoff *et al.* was specially devised to give easy access to Cyclotheonamide analogues bearing a hydrophobic group at the α-amine of diaminopropanoic acid, by using a N^{α}-protected diaminopropanoic acid derivative which was deprotected in a late-stage in the synthesis (see **23**). Very recently, Maryanoff *et al.* described the synthesis of several Cyclotheonamide analogues using this [3 + 2] approach in combination with the late-stage deprotection protocol.[12]

Scheme 2.4. Synthesis of Cyclotheonamide A and B according to Maryanoff *et al.*.[4,11]

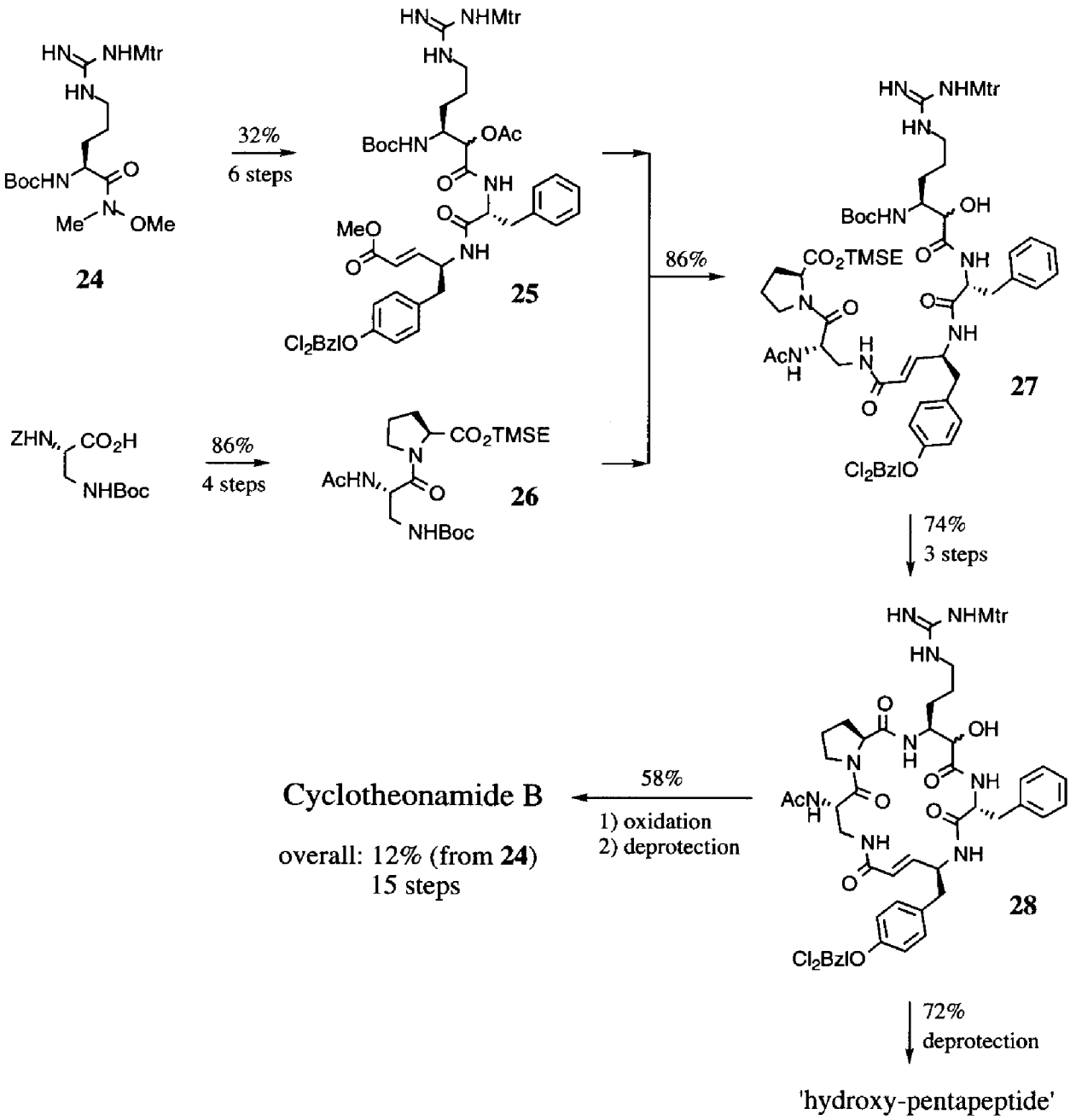


Boc: *t*-butyloxycarbonyl; Fmoc: fluorenylmethoxycarbonyl; Pht: phtaloyl; Ts: *p*-toluenesulfonyl; SEM: [2-(trimethylsilyl)ethoxy]methyl; Z: benzyloxycarbonyl; TBDMS: *t*-butyldimethylsilyl.

The most recent synthesis is that of the Shiori group (Nagoya City University) in co-operation with Fusetani's group which originated the research on Cyclotheonamide (University of Tokyo).[5] It started with N^{α}-Boc, N^{ω}-Mtr-arginine *N,O*-dimethylhydroxamate (**24**), which was elegantly homologated in high yield with furyllithium as a carboxylate anion equivalent to an α-hydroxy-β-homoarginine derivative, and elaborated into tripeptide **25** with a protected α-hydroxyl group [Scheme 2.5].

Condensation of key intermediates **25** and **26** gave linear pentapeptide **27**, which was macrolactamized in a three-step procedure to furnish cyclopentapeptide **28** in 74% yield. After oxidation and final deprotection, Cyclotheonamide B was obtained in 58% yield from the protected cyclopentapeptide **28**. Of all four syntheses, Shiori's one is the most efficient with an overall yield of 12% starting from **24**. The two hydroxy epimers of cyclopentapeptide **28** were separately deprotected and isolated, and also tested for their thrombin inhibiting properties [Chapter Seven].

Scheme 2.5. Synthesis of Cyclotheonamide B according to Shiori *et al.*.[5]



Mtr: 2,5,6-trimethyl-4-methoxybenzenesulfonyl; Boc: *t*-butyloxycarbonyl;
Ac: acetyl; Cl_2Bzl: 2,6-dichlorobenzyl; TMSE: (2-trimethylsilyl)ethyl.

2.3. Strategic Considerations

A typical synthetic plan to a complex molecule such as Cyclotheonamide is a matrix of several inter-dependent and parallel strategies encompassing such issues as fragment synthesis, fragment linkage, stereochemistry, functional group interconversion, and protecting groups. Particularly in the synthesis of (cyclic) peptides, the choice of proper protection for carboxyl groups, amino groups, and side chain functional groups of the constituent amino acids is of vital importance for success. However, each protecting group lengthens a synthesis by at least two steps with the inevitable reduction in yield and increase in cost. Besides this, cleavage of a protecting group in a sensitive molecule is often a capricious matter.

The initial step in the development of a synthetic plan involves a retrosynthetic analysis, which reduces a complex molecule to simple starting materials. In Cyclotheonamide, total disconnection obviously and inevitably leads to the five constituent amino acids [Chapter 1.4, Scheme 1.5] as starting materials for an efficient, straightforward synthesis. The sequence of disconnections in Cyclotheonamide is dictated by tactical considerations regarding the construction of the synthetic target, *e.g.* a linear versus a convergent approach.[18]

Obviously, the information presented in Section 2.2 was not available when we started our studies in 1991. At that moment, the following strategic considerations led to our synthetic plan towards Cyclotheonamide and analogues.

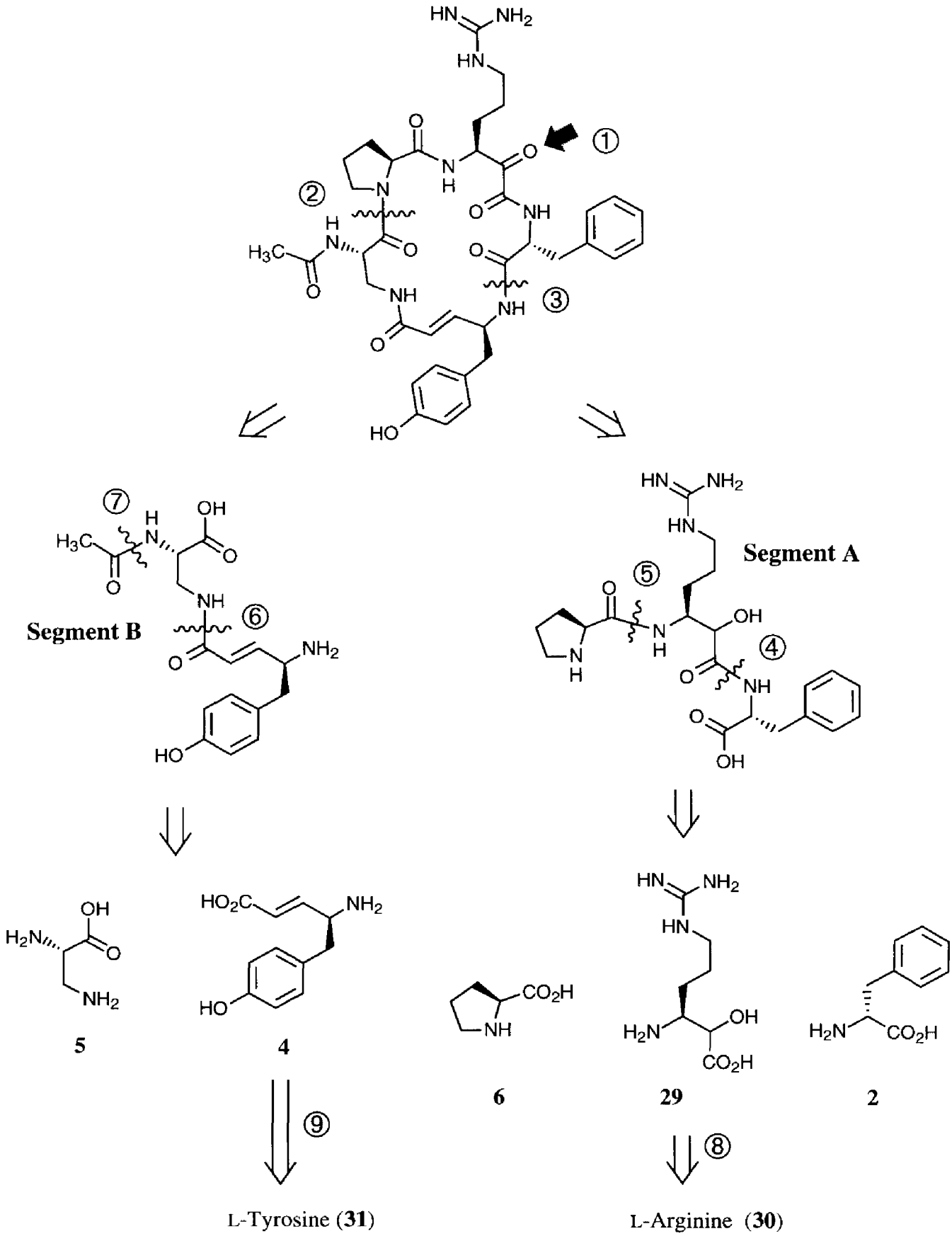
2.3.1. Retrosynthesis

It was anticipated that the electrophilic keto group of α-oxo-β-homoarginine would be very difficult to preserve during the entire synthesis. Therefore, it was decided that the α-keto amide was to be generated from α-hydroxy-β-homoarginine in a late step in the total synthesis. Thus, interconversion of the keto group to a hydroxyl group is the first step in our retrosynthetic analysis [Scheme 2.6, step ①].

As we planned to develop a concise and flexible synthesis for both Cyclotheonamide B and analogues, a convergent approach using two key intermediates, a tripeptide and a dipeptide, was selected. A choice for one of the five possible sets of a tripeptide and a dipeptide might have been made upon synthetic considerations, *e.g.* the desire to suppress racemization of the *C*-terminal amino acid residues during segment condensation and macrocyclization by employing activated amino acid residues which are less prone to racemization such as vinylogous tyrosine and proline. However, we selected a tripeptide and a dipeptide on the basis of a molecular modelling study of Cyclotheonamide and thrombin.[19] In this study we found that the Pro–kArg–D-Phe sequence of Cyclotheonamide interacted well with the active site of thrombin, *viz.* the proline ring occupied the S_2 pocket and the arginine side chain resided in the S_1 pocket of thrombin (see also Figure 1.1). Serine-195 of thrombin was optimally located to form a hemiketal with the keto group of the ligand.[20] The aromatic side chain of D-phenylalanine was located in a lipophilic region of the enzyme. The vTyr–Dpr moiety, however, did not seem to contribute much to the binding of the natural product to thrombin as the hydroxyphenyl group appeared to be exposed to the solvent, and the formyl or acetyl substituent of the diaminopropanoic acid part was not able to occupy the large hydrophobic S_3 pocket.

Later on, this mode of interaction was largely confirmed and refined by the X-ray diffraction study of Tulinsky involving the Cyclotheonamide A-thrombin-hirugen complex.[4,11,12]

Scheme 2.6. Retrosynthetic analysis of Cyclotheonamide B.



As a result of these modelling studies we anticipated that modification of the vTyr–Dpr dipeptide, *e.g.* by stripping the vTyr side chain and/or introduction of a more hydrophobic acyl group on Dpr, might have a positive effect on the potency and selectivity towards thrombin inhibition. So, by employing a synthetic route with Pro–kArg–D-Phe as a common intermediate and modifying the two other amino acid residues, we would be able to conveniently prepare Cyclotheonamide A/B, as well as a set of analogues. Furthermore, in our view, the linear pentapeptide Pro–kArg–D-Phe–vTyr–Dpr would be interesting from a biological point of view, as the activity of it would possibly clarify the effect of the ring closure on the biological activity.

Thus, disconnection of the Dpr–Pro amide bond (step ②) gives the desired linear sequence of amino acids, and subsequent breaking of the D-Phe–vTyr bond (step ③) results in the two desired key intermediates: the tripeptide Pro–hArg–D-Phe, *i.e.* Segment A, and the dipeptide vTyr–Dpr, *i.e.* Segment B.

Further disconnection of the amide bonds of Segment A and B, gives five amino acids (step ④ to ⑦). The three α-amino acids L-proline (**6**), D-phenylalanine (**2**), and L-2,3-diaminopropanoic acid (**5**), are commercially available. Vinylogous tyrosine (**4**) and α-hydroxy-β-homoarginine (**29**) can also be reduced to commercially available α-amino acids, *viz.* L-tyrosine (**31**) and L-arginine (**30**) (step ⑧ and ⑨). A more elaborate retrosynthetic analysis for hArg and vTyr is given in the Chapters Three and Four, respectively.

2.3.2. Synthetic considerations and protecting group strategy

Once we had selected the two key intermediates, their synthesis and their elaboration into the target compound, as well as the selection of the protecting groups had to be addressed.

The presence of two highly functionalized, non-proteinogenic amino acids in our key intermediates, *viz.* α-hydroxy-β-homoarginine and a vinylogous tyrosine, makes the proper choice for a orthogonal set of protecting groups especially important. In order to establish a concise and efficient route to Cyclotheonamide and analogues, a minimal set of orthogonal protecting groups would be desirable.[21] Furthermore, in order to add some flexibility to our strategy, in case a particular pathway would become unwieldy or nonviable, the protecting groups used should allow cleavage, or even, if necessary, exchange for different protecting groups at different stages in the synthesis.

When considering Segment A, the synthetically most challenging part is the α-hydroxy-β-homoarginine residue (**29**) [Scheme 2.6]. Arginine is probably the most notorious of all natural amino acids and the synthesis of arginine-containing peptides is often very cumbersome due to problems with the (protected) guanidino group of the arginine side chain.[22] Insufficient stability, lactam formation, incomplete cleavage of the protecting groups and many other side reactions are observed. In our study, additional problems were expected to arise as it was foreseen that successful homologation of arginine to α-hydroxy-β-homoarginine would be very dependent on proper protection of the guanidino group.

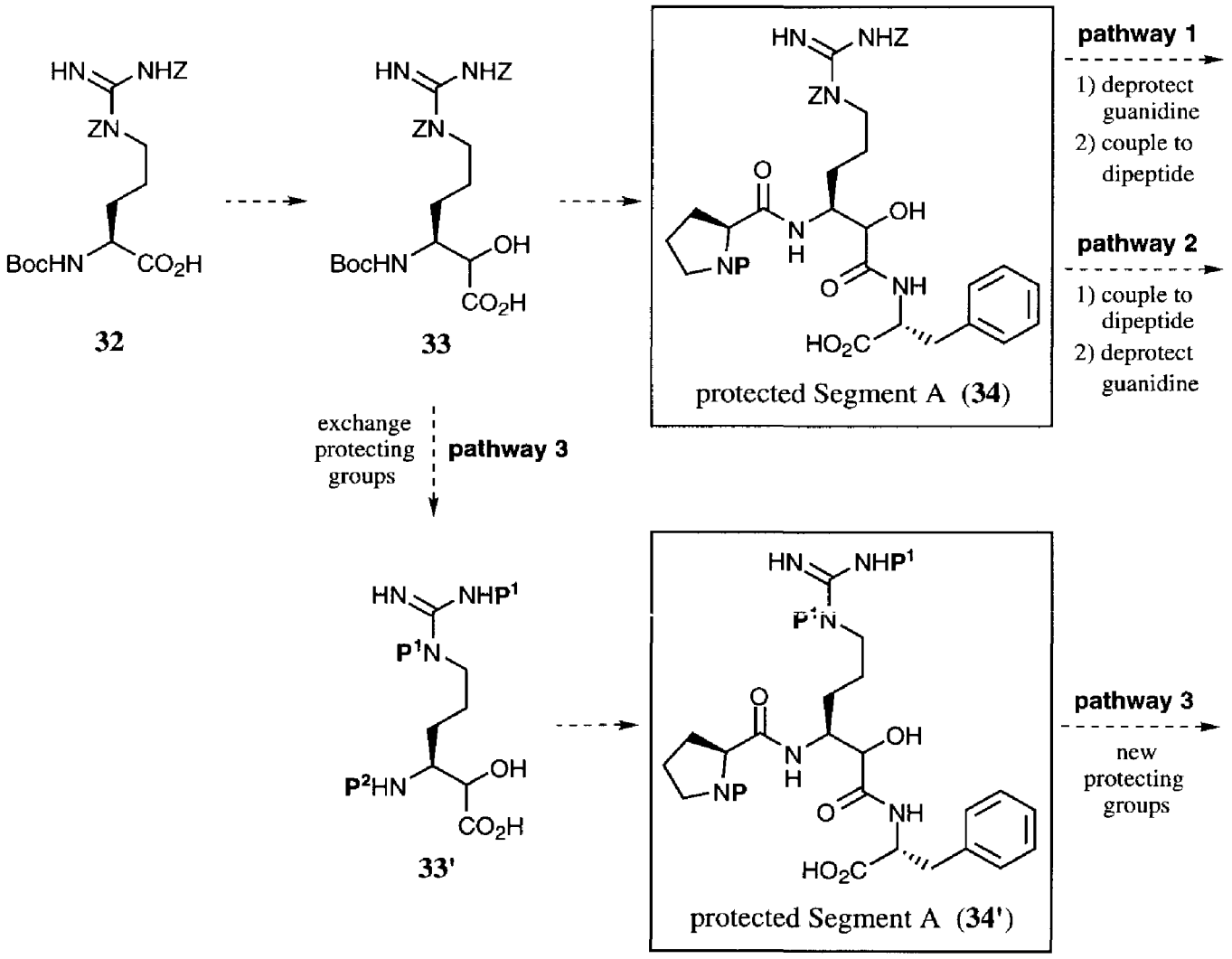
The orthogonally protected N^{α}-Boc, $N^{\delta,\omega}$-(bis-Z)arginine (**32**) [Scheme 2.7] was initially selected as starting material for the arginine homologue as we expected that the presence of two Z groups at the guanidino moiety would give effective protection during homologation procedures. Furthermore, cleavage of the Z group is possible under both neutral (H_2/Pd) and acidic (trifluoroacetic acid) conditions,[23] important for the desired flexibility of our strategy (*vide infra*). Finally, the synthesis of **32** by a recent literature procedure was rather convenient, and the preparation of large amounts of starting material was found to be fast and inexpensive.[24]

The product of the homologation reaction of **32**, *i.e.* N^{β}-Boc, $N^{\varepsilon,\omega}$-(bis-Z)-α-hydroxy-β-homoarginine (**33**) was expected to be readily extended by peptide coupling, preferably without protection of the hydroxyl group, to give **34**, a protected derivative of Segment A.

Deprotection of the bis-Z guanidino unit in **34** by hydrogenolysis (neutral conditions) has to be performed prior to fragment condensation to prevent reduction of the alkene moiety of the vinylogous tyrosine unit in Segment B [Scheme 2.7, pathway 1]. However, if hydrogenolytic removal of the Z groups is impossible or if the presence of the unprotected guanidino unit appears to be incompatible with the intended reaction sequence for completing the synthesis of Cyclotheonamide B, the Z groups might also be removed at a later stage by treatment with trifluoroacetic acid (pathway 2).

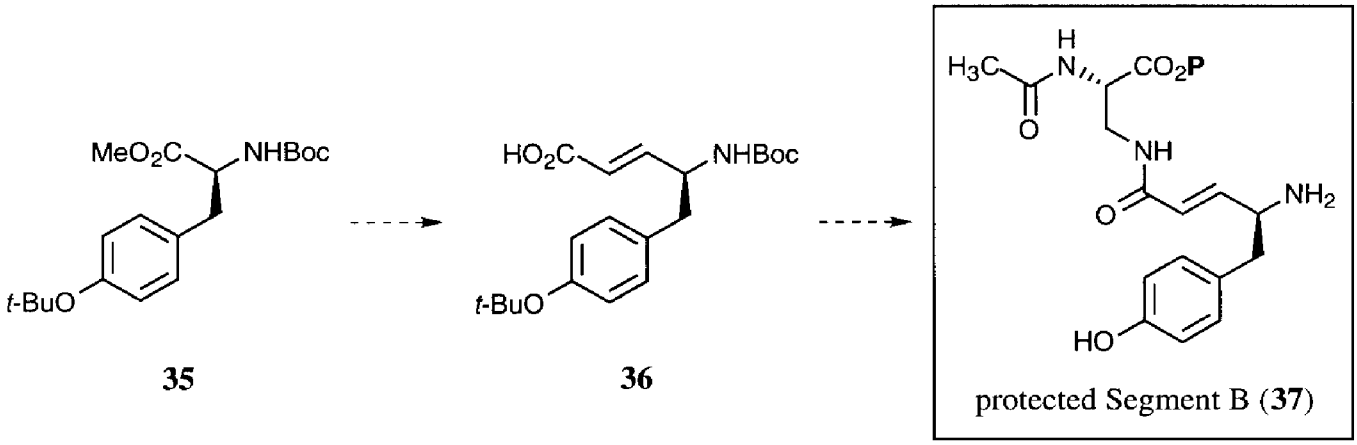
If both approaches prove to be unsuccessful, the Z groups at the arginine side chain have to be exchanged for different protecting groups (**33**→**33'**), preferably in an α-hydroxy-β-homoarginine derivative before tripeptide formation (pathway 3). In that case, it might be considered appropriate, especially for the efficient synthesis of analogues, to devise a new synthesis of α-hydroxy-β-homoarginine **33'** employing the new protecting group strategy right from the start.

Scheme 2.7. Approach to protected Segment A (**34**); a flexible protecting group strategy.



It was foreseen to prepare Segment B by starting from easily available **35** containing acid-labile *O,N*-protecting groups [Scheme 2.8]. A Wittig-type conversion of **35** should yield vinylogous tyrosine **36**. Coupling of the latter with a suitable 2,3-diaminopropanoic acid derivative and acidic treatment to cleave both the *t*-butyl ether and Boc group should produce **37**, a protected derivative of Segment B.

Scheme 2.8. Approach to protected Segment B (**37**).



35 **36** protected Segment B (**37**)

Deprotection of the hydroxyphenyl group at this stage obviously implies that late-stage oxidation of the secondary hydroxyl group of the β-homoarginine unit is compatible with the presence of a free phenolic hydroxyl group. However, in case this selective oxidation is not feasible, selective deprotection of (derivatives of) **36** would have to be investigated or a synthesis of a tyrosine derivative with orthogonal *O,N*-protection, allowing selective deprotection of the amino group in the dipeptide, would have to be devised.

Furthermore, the two protecting groups **P** in **34** and **37** should preferably be of the same category, thus allowing simultaneous deprotection of the *C*- and *N*-terminus in the linear pentapeptide. Sets of protecting groups that meet this requirement are: *t*-butyl and Boc, 2-(trimethylsilyl)ethyl (TMSE) and 2-(trimethylsilyl)ethoxycarbonyl (Teoc), or allyl and allyloxycarbonyl (Aloc).

Finally, two consequences of the favoured [3 + 2] strategy should not be neglected. Firstly, in the coupling reaction of Segment A with Segment B, the former segment has to be activated at the *C*-terminus. This might lead to δ-lacton formation by nucleophilic attack of the hydroxyl group of the β-homoarginine unit and could necessitate protection. Secondly, cyclization of the linear pentapeptide requires attack by the proline amino group with its moderate nucleophilicity as compared to amino groups of the other amino acids.

2.4. Conclusions

In this chapter a convergent [3 + 2] fragment-condensation approach to Cyclotheonamide B and analogues is set out. Two key intermediates have been defined, *i.e.* Segment A, a tripeptide containing an α-hydroxy-β-homoarginine unit, to be synthesized from arginine derivative **32**, and Segment B, a dipeptide in which a vinylogous tyrosine, to be synthesized from tyrosine derivative **35**, is present.

The selection of the key fragments is based upon a retrosynthetic analysis in which practical (*e.g.* availability of starting materials) and theoretical (molecular modelling studies) considerations are combined. Segment A, containing the amino acid residues important for the interaction with thrombin can serve as a common intermediate for the synthesis of Cyclotheonamide A/B and analogues, as well as for biologically interesting linear peptides.

2.6. References and Notes

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18. The overall yield in a synthetic sequence is the product of the yields of the individual steps; so the total yield tends to decrease with an increasing number of steps in a sequence. A linear sequence maximizes the number of steps to which the original starting materials must be subjected. A convergent synthesis, in contrast, allows one to build up separate fragments and then combine them; the number of steps to which each starting material is subjected is thus decreased. Furthermore, analogues are more easily accessible through a common fragment, rather then starting the whole reaction sequence from the starting materials for each analogue.

19. These studies were carried out by P.D.J. Grootenhuis at N.V. Organon, Oss, The Netherlands. At the time these studies were performed, the X-ray studies presented in Chapter One were not available yet.
20. The chymotrypsinogen numbering is used to designate the residues in thrombin.
21. An orthogonal set of protecting groups is *ideally* a set of protecting groups whose members can be manipulated in any order, without effecting the other members.
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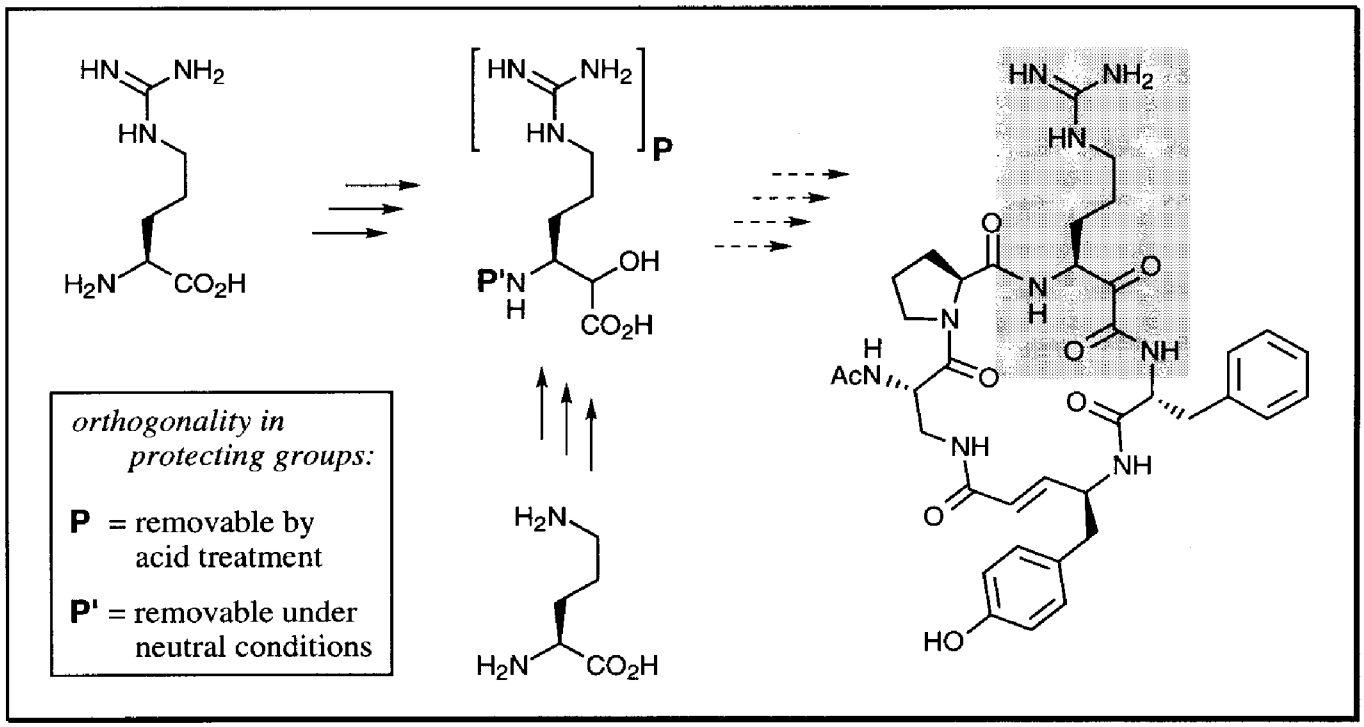
CHAPTER THREE

Synthesis and Properties of α-Hydroxy-β-Homoarginine Derivatives



Abstract

In this chapter we present two syntheses of α-hydroxy-β-homoarginine derivatives starting with arginine or ornithine. Proper protection of the guanidino group and careful control of the homologation conditions were found to be very critical to the ultimate success.

Several α-hydroxy-β-homoarginine derivatives were used as model compounds to validate the different strategic alternatives set out in Chapter Two. The results of these model studies prompted us to design a β-homoarginine building block having guanidine protecting groups which are removable by mild acid treatment.

This new β-homoarginine derivative would prove to be excellently suitable for the synthesis of Cyclotheonamide B and analogues.

3.1. Introduction

The most distinct feature of Cyclotheonamide is, as set out in Chapter One, the modified amide bond between arginine and D-phenylalanine. This α-keto amide unit in question plays a predominant role in the mechanism of enzyme inhibition *via* formation of a highly stabilized hemiketal [Chapter 1.4]. Also from a synthetic point of view, the presence of this electrophilic α-keto carboxylic acid moiety, in combination with the very basic and nucleophilic guanidine part and the unusual and racemization-prone L-2,3-diaminopropanoic acid residue, makes Cyclotheonamide a challenging target worthwhile to be studied.

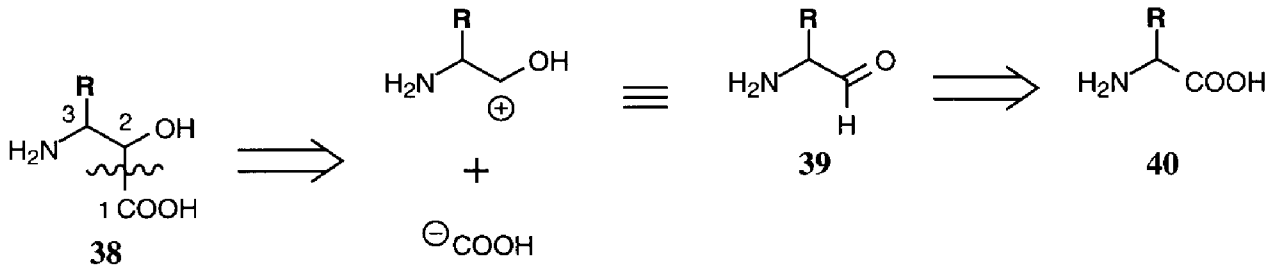
Although several elegant and direct routes to α-keto carboxylic acids are known[1] we decided that, due to the high reactivity of α-keto carboxylic acid derivatives in general and of derivatives of arginine in particular, this unit was to be formed by oxidation of an α-hydroxy-β-homoarginine unit (an α-hydroxy-β-amino acid) in a late step in the total synthesis [Chapter 2.3.1].

3.1.1. α-Hydroxy-β-amino acids

A large variety of synthetic routes to α-hydroxy-β-amino acids, *i.e.* **38**, has been devised as they are important constituents of a number of natural products and (potential) drugs *e.g.* α-hydroxy-β-homophenylalanine (**38**, **R** = CH_2Ph; 2*S*,3*R*) is found in bestatine,[2] α-hydroxy-β-homophenylglycine (**38**, **R** = Ph; 2*R*,3*S*) is part of taxol,[3] and (2*R*,3*S*)-3-amino-4-cyclohexyl-2-hydroxybutyric acid (**38**, **R** = $CH_2(C_6H_{11})$) is present in a series of renin inhibitors.[4,5]

One of the many possibilities for constructing these α-hydroxy-β-amino acids is derived from the retrosynthetic analysis in Scheme 3.1, starting with α-amino acids **40** as expedient starting materials.

Scheme 3.1. Retrosynthetic analysis of α-hydroxy-β-amino acids.



In this analysis, disconnection of the C_1-C_2 bond in **38** results in a nucleophilic $^-$COOH unit and α-amino aldehyde **39**, which is readily accessible from α-amino acid **40**. As many α-amino acids are commercially available, a synthetic route based on this analysis is easily applicable to a wide variety of side chains (**R**) and to both stereoisomers of the C_3-centre of **38**.

Thus, the first step in the homologation of an α-amino acid is the preparation of a protected aldehyde **42** [Scheme 3.2, step 1]. Chiral α-amino aldehydes are important synthetic building blocks for the synthesis of natural and synthetic products. Two methods for their preparation are frequently used. Reduction of the methyl ester of a protected α-amino acid, *e.g.* **41**, $R^1 = OCH_3$, with diisobutylaluminium hydride (DiBAH) at low temperature, followed by mild acidic work-up is most in vogue.[6] A useful alternative, which is becoming increasingly popular, is reduction of a Weinreb amide,

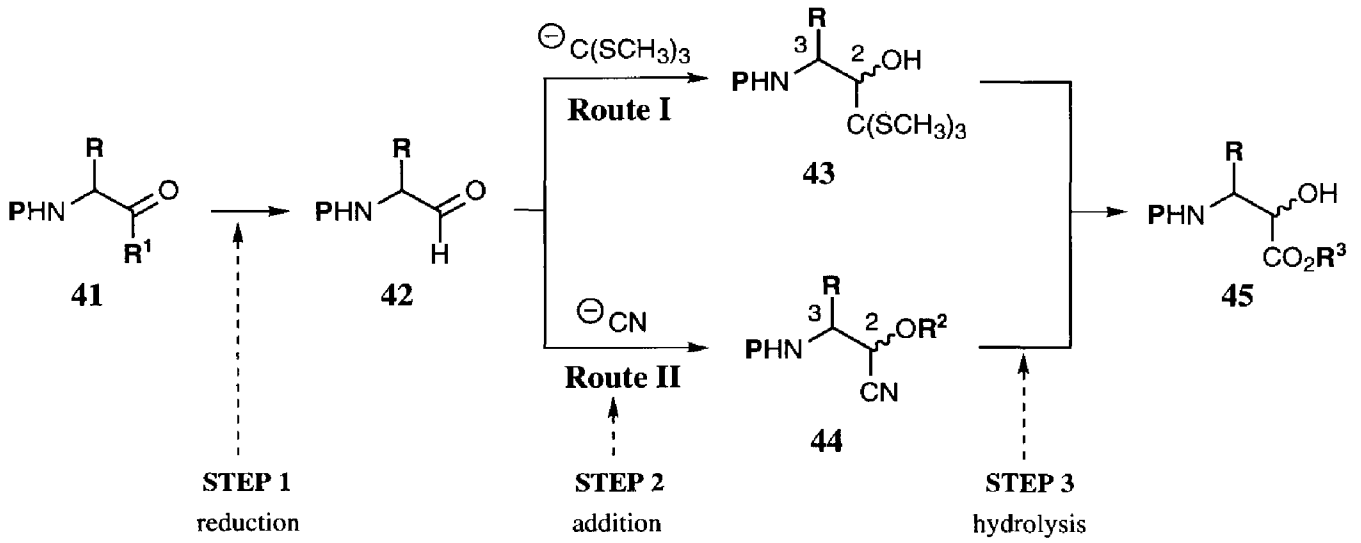
e.g. **41**, $\mathbf{R^1}$ = $N(OCH_3)CH_3$, with $LiAlH_4$.[7] As the chiral α-amino aldehydes **42** are susceptible to racemization by keto-enol tautomerism, they are generally not purified but, after a fast work-up, used as such.

Upon the addition of a $^-$COOH synthon to **42**, a second chiral centre (C_2) is generated, so that in principle two diastereoisomers can be formed [Scheme 3.2, step 2]. In the projected synthesis of Cyclotheonamide B, this newly formed chiral centre is oxidized eventually, thus the formation of epimers is irrelevant in this particular case. However, formation of epimers leads to a mixture of compounds with different physical properties (which might hamper purification and characterization) and a possible difference in reactivity.

In Scheme 3.2 two popular $^-$COOH synthons are depicted, *viz.* $^-C(SCH_3)_3$ (Route I) and $^-$CN (Route II). The usefulness of tris(methylthio)methyllithium as a carboxylate anion equivalent (Route I) was recognized by Seebach in the late sixties.[8] Recently, his method has been applied to the synthesis of N^{α}-Boc, N^{ω}-Z-α-hydroxy-β-homolysine methyl ester (**45**, **P** = Boc, **R** = $(CH_2)_4NHZ$ and $\mathbf{R^3}$ = CH_3).[9] Thus, addition of tris(methylthio)methyllithium, at low temperature, to the protected lysine-derived α-amino aldehyde **42** (step 2, Route I) and subsequent Hg^{2+}-mediated hydrolysis of α-hydroxy orthothioester **43** (step 3), under neutral conditions ($HgCl_2$/HgO/MeOH/H_2O), gave α-hydroxy methyl ester **45**. Depending on the exact conditions for hydrolysis, either an α-hydroxy ester or an α-hydroxy carboxylic acid can be prepared.[8]

Also the preparation of α-hydroxy-β-amino acids *via* cyanohydrin intermediates has already been reported.[10,11] Cyanohydrins **44** ($\mathbf{R^2}$ = H) can be obtained by reaction of an α-amino aldehyde with an appropriate cyanide source, *e.g.* sodium cyanide, lithium cyanide, or trimethylsilyl cyanide (step 2, Route II). Acidic hydrolysis (concentrated HCl_{aq}, ΔT) of cyanohydrin acetate **44** (**P** = isopropyloxycarbonyl, **R** = CH_2Ph, and $\mathbf{R^2}$ = Ac), prepared from the corresponding aldehyde and sodium cyanide/acetic anhydride under phase transfer conditions, furnished α-hydroxy-β-homophenylalanine·HCl (**45**, **P** = H, **R** = Ph, and $\mathbf{R^3}$ = H).[10] Hydrolysis under milder acidic conditions is also possible: treatment of a cyanohydrin with dry methanolic hydrogen chloride at 4 °C, followed by treatment with H_2O gives α-hydroxy methyl esters.[11,12]

Scheme 3.2. Three step sequence for the preparation of α-hydroxy-β-amino acids from α-amino esters.



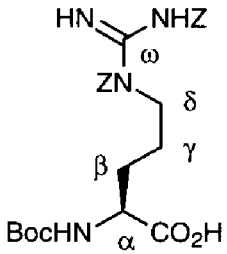
The protecting group **P** in **41**, and the side chain **R** (which, if functionalized, is also protected) strongly influence the choice of the nucleophile to be used in step 2. The nucleophile may exhibit undesirable reactivity towards the α-amine protecting groups or the protected side chain; furthermore, the conditions for hydrolysis (step 3) should be compatible with the functional and protecting groups present. Route I seems to be more generally applicable as the neutral (oxidative) conditions for hydrolysis of the attached synthon are less extreme than the acidic conditions necessary in Route II, and are therefore compatible with a wider variety of functional and protecting groups.

3.1.2. Arginine and α-hydroxy-β-homoarginine

In this chapter the synthesis and properties of α-hydroxy-β-homoarginine derivatives are described. At the start of our investigations, homologation of arginine to α-hydroxy-β-homoarginine had not yet been described in the literature.

Particularly for the homologation of arginine, proper protection of the highly functionalized starting material, *i.e.* arginine, was considered to be very important for the ultimate success.

The use of only one strong electron-withdrawing group is often sufficient in blocking the very basic and nucleophilic guanidino function for performing peptide synthesis, in solution as well as in the solid phase. In our arginine derivative selected as starting material, guanidine protection should not only be effective during peptide synthesis but should also withstand homologation to α-hydroxy-β-homoarginine. Therefore, we decided to fully block the arginine side chain, using at least two protecting groups. This would also decrease the polarity of the side chain and thereby enhance the solubility in organic solvents and improve the chromatographic properties of the arginine derivatives. As we anticipated that Boc and Z groups were compatible with the reaction conditions needed for the preparation of α-hydroxy-β-amino acids *via* an orthothioester intermediate (Route I, Scheme 3.2), arginine **32**, easily accessible by a new procedure,[13] was selected as starting material.



32

Unfortunately, the $N^{\delta,\omega}$-(bis-Z) protected guanidino group of **32** proved to be very sensitive towards carbanions and basic conditions in general. Thus attempt towards homologation of **32** *via* Route I with tris(methylthio)methyllithium were unsuccessful [Section 3.2.1].

Difficulties were also encountered during homologation *via* cyanohydrin formation (Route II). However, by careful control of reaction conditions, the initially observed cleavage of a Z group upon cyanohydrin formation could be suppressed nearly completely [Section 3.2.2]. Nevertheless, under the basic conditions necessary for elaboration of the arginine homologue into more advanced Cyclotheonamide intermediates, the Z group was rapidly cleaved. This time, the loss of this Z group could not be prevented, not even under mild basic conditions [Section 3.2.3].

To study the feasibility of the three pathways set out in our synthetic plan in Scheme 2.7 [Chapter

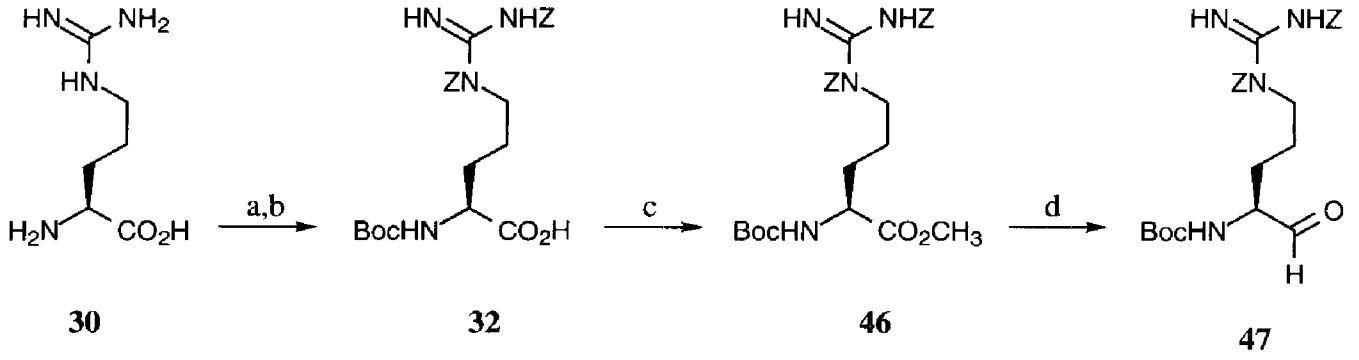
Two], some α-hydroxy-β-homoarginine derivatives prepared *via* the cyanohydrin route were used as model compounds [Section 3.2.3]. It was found that the presence of at least one Z group at the guanidino moiety was necessary to allow oxidation of the α-hydroxy carboxylic acid unit. However, we were unable to cleave this Z group by acid treatment in the resulting keto-arginine derivative. Therefore, it was decided to exchange the guanidine protecting groups for groups that are more acid-labile. These new protecting groups were introduced in an α-hydroxy-β-homoarginine derivative [Section 3.3], and in an arginine derivative [Section 3.4]. The latter was successfully homologated *via* an orthothioester intermediate (Route I, Scheme 3.2).

3.2. Preparation of α-Hydroxy-β-Homoarginine from N^{α}-Boc, $N^{\delta,\omega}$-(bis-Z)Arginine (32): Problems and Solutions

3.2.1. Attempted homologation of 32 *via* an orthothioester intermediate (Route I)

Our first attempt was based on the reported homologation of N^{α}-Boc, N^{ε}-Z-lysine. Thus, **32**[13] prepared from arginine (**30**) was treated with diazomethane[14] to furnish methyl ester **46** [Scheme 3.3], which was subjected to the tris(methylthio)methyllithium based homologation procedure described in Section 3.1. Ester-reduction of **46** with DiBAH, to yield **47** proceeded sluggishly. The best results were obtained by employing 3.5 equiv of DiBAH at –63 °C during 75-90 min, to give amino aldehyde **47** in 92% yield.

Scheme 3.3. Preparation of N^{α}-Boc, $N^{\delta,\omega}$-(bis-Z)arginal **47**.

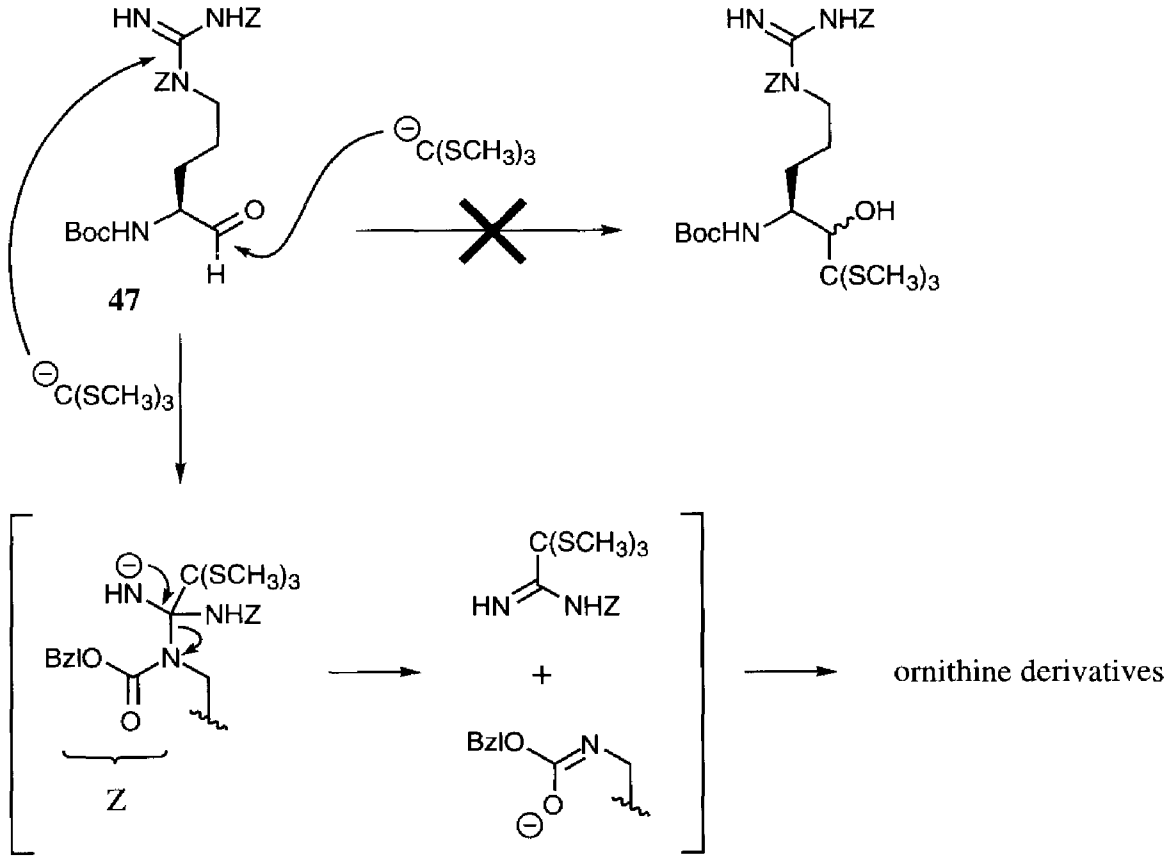


30 **32** **46** **47**

a) Boc_2O, H_2O/1,4-dioxane, 16 h, 100%; b) TMS-Cl/DiPEA, 1,2-dichloroethane, 43 °C, 1.5 h, then, Z-Cl/DiPEA, 0 °C → rt, 4 h, 63%; c) CH_2N_2, CH_2Cl_2/EtOH, 0 °C, 95%; d) DiBAH, CH_2Cl_2, –65 °C, 75-90 min, 92%.

The crude aldehyde **47** was immediately dissolved in dry tetrahydrofuran (THF), cooled to –65 °C, and subsequently allowed to react with tris(methylthio)methyllithium. The ^{1}H-NMR spectrum of the worked-up reaction mixture was too complex to allow complete interpretation. However, it was concluded that the starting material had been consumed (no aldehyde resonance was observed). Compared to the ^{1}H-NMR spectrum of aldehyde **47** or methyl ester **46**, the characteristic δ-H's were shifted highfield for about 0.8 ppm. From this we inferred that the organolithium compound had attacked the electrophilic carbon atom of the protected guanidine group, which resulted in de-amidation to an ornithine derivative [Scheme 3.4].

Scheme 3.4. Proposed mechanism for de-amidation of **47** by tris(methylthio)methyllithium.



Attempts to overcome this (presumed) de-amidation reaction failed. The reaction of aldehyde **47** with α-(bromomagneso)-ethylvinyl ether,[15] another very nucleophilic $^{-}$COOH synthon, suffered from the same drawback. At this point it became clear that carbanion chemistry was not compatible with the guanidine bis-Z protecting group strategy and so Route I [Scheme 3.2] had to be abandoned. Having reached this conclusion, we focused on the preparation of α-hydroxy-β-homoarginine from **32** *via* Route II, employing the less nucleophilic cyanide.

3.2.2. Homologation of 32 *via* a cyanohydrin acetate intermediate (Route II)

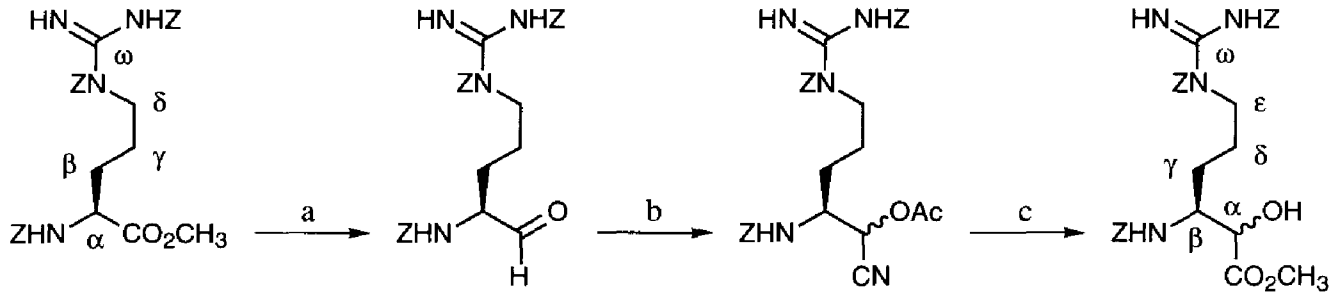
At the onset of our studies towards the feasibility of Route II, we reasoned that it was a prerequisite to preserve the amine protecting group **P** during the conversion of nitril **44** into carboxylic acid derivative **45** [Scheme 3.2]. Based on this line of reasoning we abandoned the N^{α}-Boc protected starting material **32**. However, before we embarked on a search for another, proper N^{α} protecting group, we wanted to ascertain that Route II was in principle a viable one. Therefore, the readily available $N^{\alpha,\delta,\omega}$-(tris-Z)-arginine methyl ester (**48**) was used as a model compound,[16] whereby we realized that the homologation product of **48** was inappropriate for pursuing the synthesis of Segment A, as it lacked the required orthogonal protection.

Reduction of **48** with DiBAH yielded aldehyde **49** in good yield [Scheme 3.5]. The reaction of **49** with NaCN/Ac_2O in H_2O/CH_2Cl_2, containing a phase-transfer catalyst, was terminated after 3 h at room temperature.[10] Analysis of the ^{1}H-NMR spectrum of the crude cyanohydrin acetate, in combina-

tion with GC-MS analysis of the volatiles (containing benzyl acetate and benzyl alcohol) showed that under these conditions the N^{δ}-Z group had been cleaved extensively. Fortunately, formation of the cyanohydrin acetate proved to be much faster than N^{δ} deprotection; thus, by shortening the reaction time to 40 min and lowering of the temperature to 0 °C, the initially observed N^{δ} deprotection could be suppressed almost completely. Cyanohydrin acetate **50** was obtained as a mixture of diastereomers (13:5) in an overall yield of 60% from **48**.

Treatment of this cyanohydrin with an etheral solution of hydrochloride and methanol at 4 °C yielded the hydrochloride of the corresponding imino ester, which was hydrolyzed *in situ* by addition of water to give the tris-Z protected derivative **51** in 49% yield. From this result we concluded that homologation of arginine derivatives *via* Route II was a practicable procedure.

Scheme 3.5. Synthesis of α-hydroxy-β-homoarginine derivatives *via* Route II.



48 **49** **50** **51**

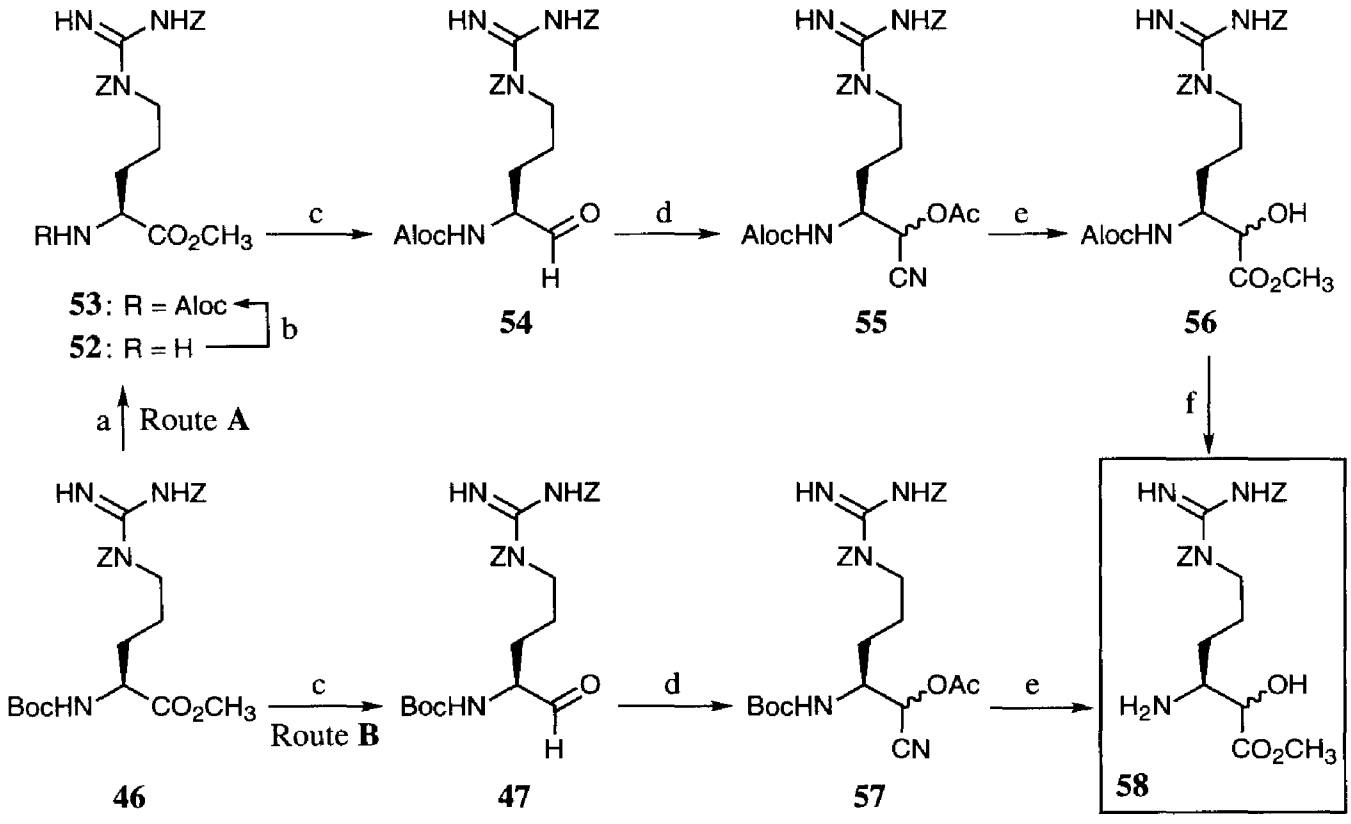
a) DiBAH, CH_2Cl_2, –65 °C, 75-90 min, 85%; b) NaCN/Ac_2O/tetrabutylammonium bromide (TBAB), CH_2Cl_2/H_2O, 0 °C, 40 min, 71%; c) HCl, Et_2O/MeOH (4:1), 4 °C, 24 h followed by H_2O, 4 °C, 1 h, 49%.

Now the stage was set to readdress the orthogonality of the arginine protecting groups. As the allyloxycarbonyl (Aloc) group was found to be stable under the acidic conditions used for hydrolysis of a cyanohydrin, N^{α}-Aloc, $N^{\delta,\omega}$-(bis-Z)arginine methyl ester (**53**) was prepared in two steps from the Boc protected methyl ester **46**, and successfully converted *via* Route II to α-hydroxy-β-homoarginine derivative **56** [Scheme 3.6, Route **A**].

Selective deprotection of the β-amino group of **56**, to facilitate coupling with proline, proceeded smoothly by treatment with tetrakis(triphenylphosphine)Pd/dimedone.[17] The crude reaction mixture was dissolved in 0.5 N HCl_{aq} and washed thoroughly with diethyl ether. After adjusting the pH of the aqueous layer with sodium carbonate (pH≈ 10), extraction with ethyl acetate gave α-hydroxy-β-homoarginine derivative **58** in 91% yield.

At this point, we realized that it might be well feasible to achieve nitril hydrolysis and *N* deprotection in one step; in other words, homologation of N^{α}-Boc protected arginine **46** might give **58** straightaway [Scheme 3.6, Route **B**]. It was gratifying to observe that this was indeed the case. Preparation of cyanohydrin **57** via aldehyde **47** was straightforward and, indeed, the *N*-Boc group was cleanly removed during hydrolysis of the cyanohydrin. Treatment of the acidic reaction mixture with a large excess of water gave a bi-phasic system from which **58** was isolated as described above. This shortcut improved the efficiency for the preparation of **58** considerably (3 steps from **46** in 55% yield, instead of 6 steps *via* **53** in 29% yield).

Scheme 3.6. Two different routes for the synthesis of α-hydroxy-β-homoarginine **58**.



a) TFA/H_2O (9:1), 45 min, 100%; b) Aloc-Cl/DiPEA, MeCN, 24 h, 94%; c) DiBAH, CH_2Cl_2, –65 °C, 75-90 min; d) NaCN/Ac_2O/TBAB, CH_2Cl_2/H_2O, 0 °C, 40 min, **57**: 60%, **55**: 72% (two steps); e) HCl, Et_2O/MeOH (4:1), 4 °C, 24 h, followed by H_2O, 4 °C, 1 h, 92%; f) $Pd(PPh_3)_4$/dimedone, THF, 45 min, 91%.

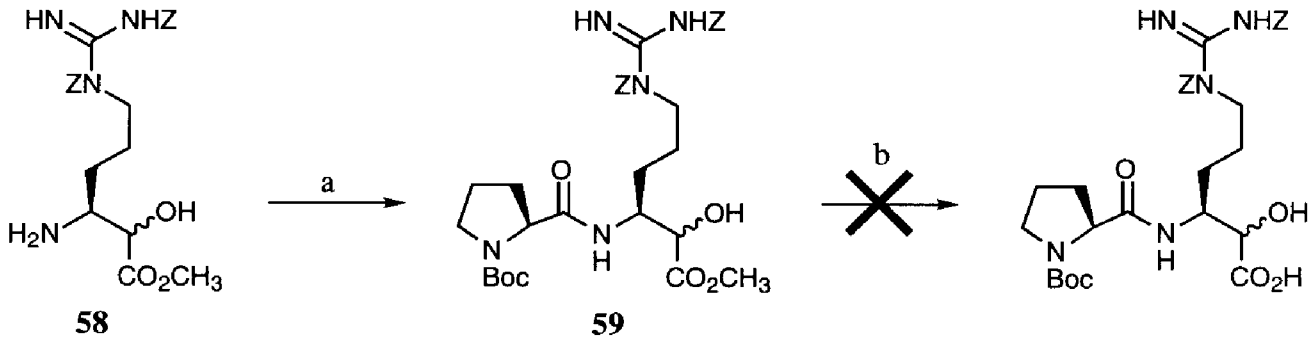
3.2.3. Properties of $N^{\varepsilon,\omega}$-(bis-Z)-α-hydroxy-β-homoarginine derivatives: deprotection and oxidation studies in model compounds

With an efficient route to α-hydroxy-β-homoarginine derivatives in hand, we were now able to study the preparation of the Pro-hArg-D-Phe tripeptide, *i.e.* Segment A. Besides being a key intermediate for the synthesis of Cyclotheonamide, we felt that this tripeptide might also serve as a model compound to validate the different synthetic alternatives set out in Chapter 2.3.

At first sight, elaboration of **58** into a tripeptide by standard protecting group handling and peptide coupling looked deceivingly simple. Coupling of **58** with *N*-Boc proline using DCC/HOBt furnished dipeptide **59** in 62% yield [Scheme 3.7]. However, saponification (LiOH 3 equiv, THF/MeOH/H_2O (4:1:1), rt, 72 min)[18] of the methyl ester of **59** did not give the expected product. The only products, isolated after aqueous work-up and unambiguously identified by ^{1}H-NMR, were *N*-Boc proline and benzyl alcohol. Formation of benzyl alcohol under these conditions for ester hydrolysis was ascribed to N^{ε}-Z group cleavage, similarly to the loss of the N^{ε}-Z group during cyanohydrin formation with **49** [Section 3.2.2]. In an effort to find reaction conditions to prevent this Z group cleavage the saponification was studied using arginine methyl ester **53** as model compound. Unfortunately, we found that even at pH= 10.3 (a pH-stat was used), the Z group was cleaved. The mechanism for this unusual Z group cleavage under these conditions is still unclear. The extreme lability of the Z group appears to be connected to its *position* at a *guanidine* unit; we never observed cleavage of the N^{ω}-Z

group of arginine, nor cleavage of the N^{δ}-Z group in ornithine derivatives under basic conditions. Moreover, this base-lability was only observed with the Z group; $N^{\varepsilon,\omega}$-(bis-Adoc)-β-homoarginine derivatives were found to be stable under basic conditions [Section 3.3].

Scheme 3.7. Attempted preparation of a tripeptide model compound from **58**.

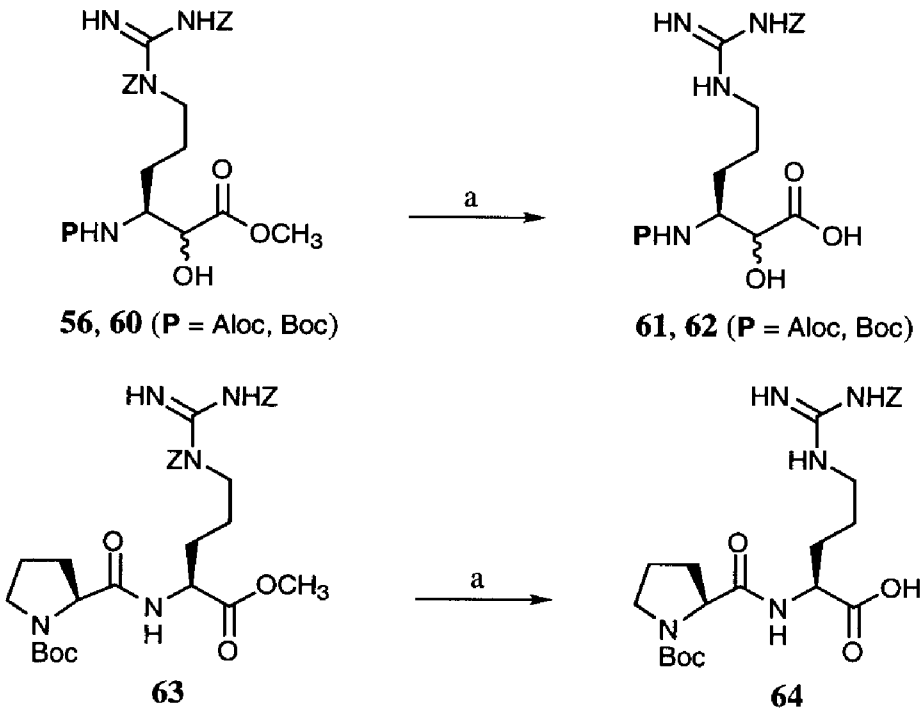


58 **59**

a) *N*-Boc proline/DCC/HOBt, THF, 18 h, 62%; b) LiOH, THF/MeOH/H_2O (4:1:1), 72 min.

A more serious problem was that ester hydrolysis of **59** was accompanied by cleavage of the amide bond, as was apparent from the isolation of *N*-Boc proline. Hydrolysis, under standard conditions, of two fully protected α-hydroxy-β-homoarginine methyl esters, *i.e.* **56** and **60** (prepared from **58** and Boc_2O)[18] gave the expected products, *viz.* **61** and **62** (note the loss of the Z group!). Also, saponification of the methyl ester of an arginine-containing dipeptide, such as **63**, did not result in cleavage of the amide bond, but gave acid **64** in excellent yield.

Scheme 3.8. Methyl ester hydrolysis of some (homo)arginine derivatives.



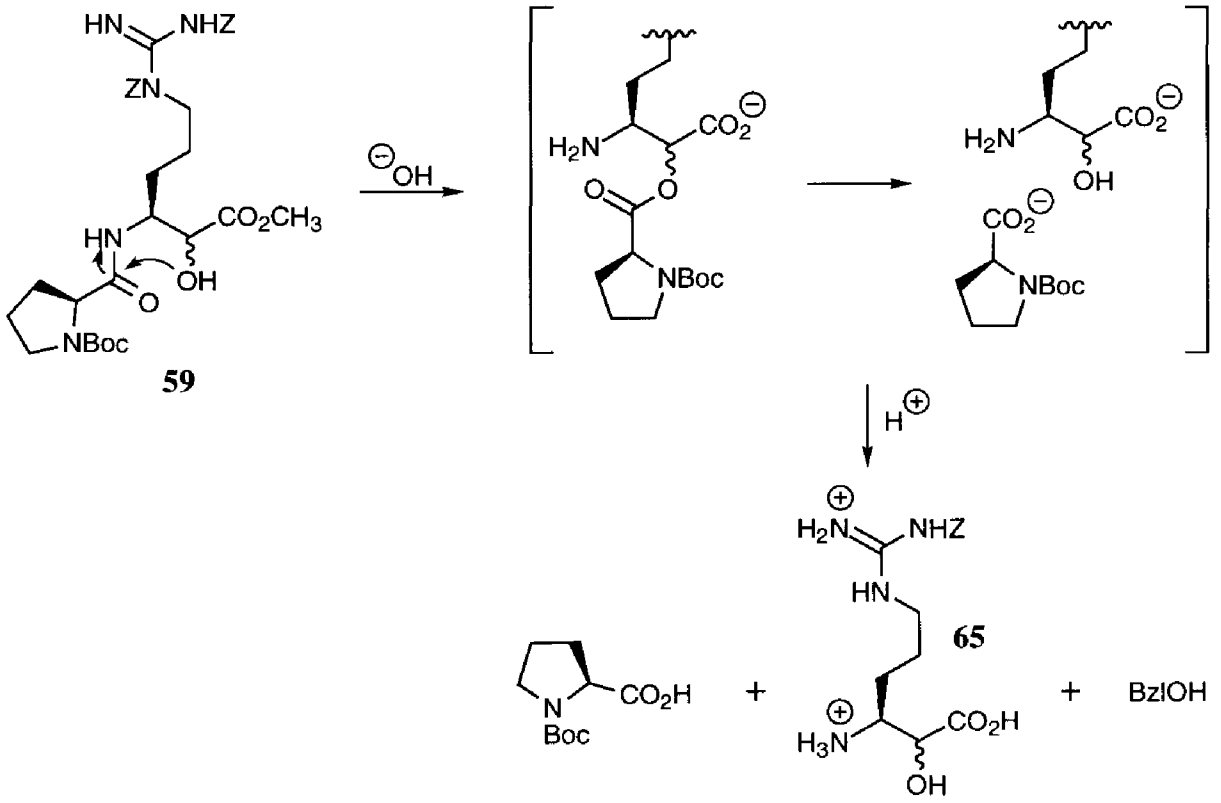
56, 60 (**P** = Aloc, Boc) **61, 62** (**P** = Aloc, Boc)

63 **64**

a) LiOH, THF/MeOH/H_2O (4:1:1), 72 min, quantitative.

Based o these findings, it was concluded that rupture of the Pro-hArg amide bond in dipeptide **59** was effecte by an intramolecular nucleophilic attack by the hydroxyl group at the amide bond as indicated in Sch ne 3.9. Presumably, migration of the proline unit followed by hydrolysis of the newly formed ester r sulted in the observed loss of the *N*-Boc proline unit. Concomitant hydrolysis of the methyl ester a l cleavage of the N^{ε}-Z group of the homoarginine part would then give **65**. This mono-protecte d β-homoarginine derivative is expected to be extremely soluble in water and, therefore, was probably removed from the reaction mixture during aqueous work-up.

Scheme 3.9. Pro-hArg amide bond cleavage in **59** by neighbouring participation.



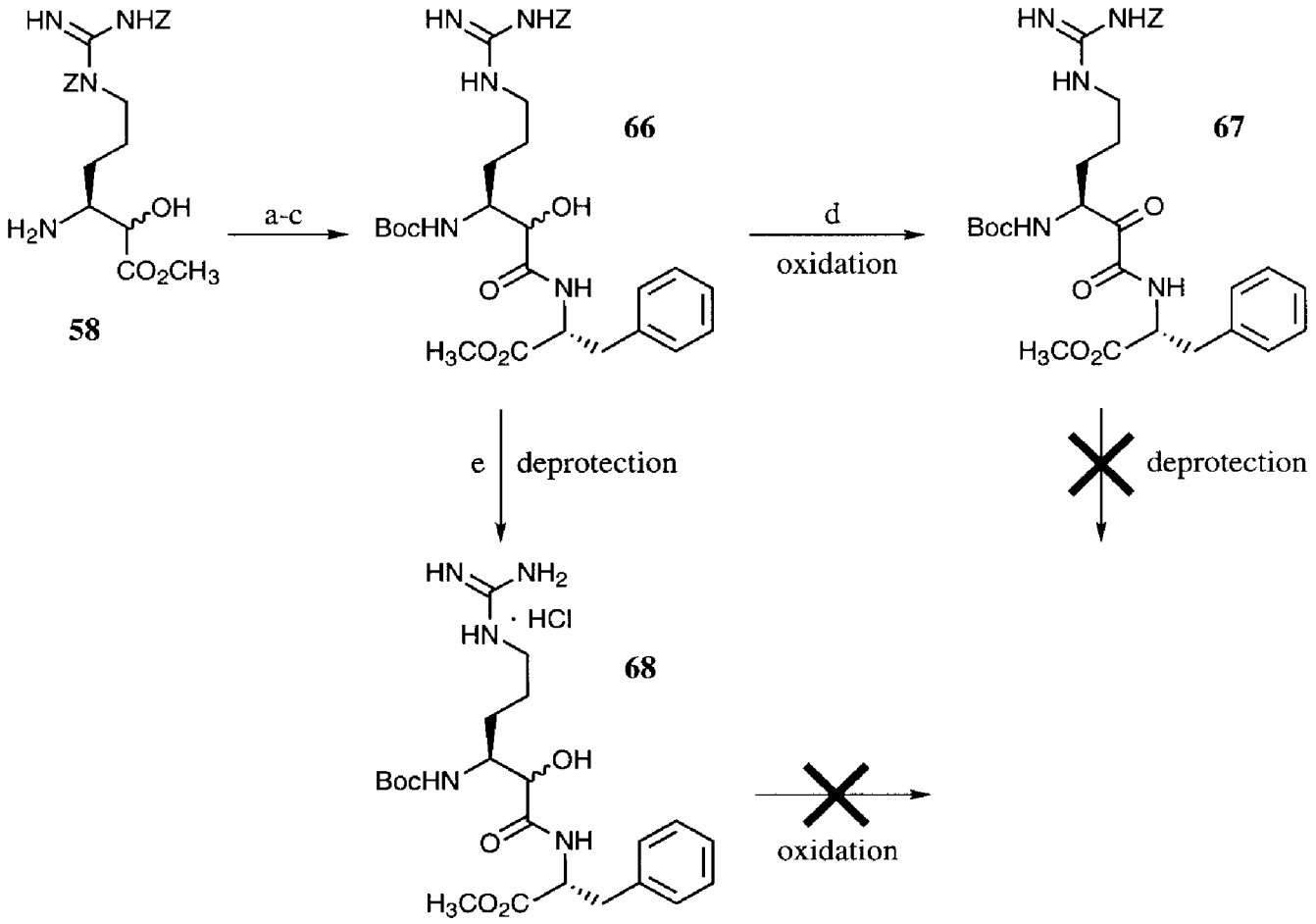
Since the hydroxyl group apparently attacks the Pro-hArg amide bond under basic conditions, blocking of this hydroxyl group might prevent cleavage of the proline unit. Introduction of a *t*-butyl-dimethylsilyl (TBDMS) protecting group in dipeptide **59** with TBDMS-Cl/imidazole[20] was rather cumbersome. However, reaction of **59** with TBDMS-trifluoromethanesulfonate/collidine[21] afforded the corresponding silyl ether quantitatively. Unfortunately, the presence of this TBDMS group prevented hydrolysis of the methyl ester; after 72 min only N^{ε}-Z cleavage was observed and upon longer reaction times also the silyl ether bond was cleaved. At this point we concluded that ester hydrolysis had to be achieved prior to peptide coupling at the β-amine of the homoarginine derivatives.

Meanwhile, dipeptide **66** had been prepared as a model compound for studying oxidation and deprotecting conditions [Scheme 3.10]. The α-hydroxy amide unit of **66** was oxidized smoothly to α-keto amide **67** with the Dess-Martin reagent.[22] Unfortunately, removal of the Z group of **67** by treatment with TFA/thioanisole[23] required several days and was accompanied by extensive degradation of the product. Probably, the presence of a protonated mono-Z protected guanidine considerably hampers

acid-catalyzed cleavage of the urethane group. The Z group lacks the strong electron-withdrawing properties of the arylsulfonyl groups which are frequently used in mono-protection of a guanidino unit and are rapidly cleaved with TFA/thioanisole.[24]

Since facile cleavage of the N^{ω}-Z group *after* oxidation proved to be very difficult, we decided to reverse the order of these reactions, *i.e. first* cleave the Z group and *then* oxidize the hydroxyl group. Unfortunately, oxidation of the hydroxyl group of dipeptide **68** (prepared by hydrogenolysis of **66**), using the Dess-Martin reagent, resulted in a complex mixture of products.

Scheme 3.10. Oxidation and deprotection of β-homoarginine dipeptides.



a) Boc_2O, DMF, 45 °C, 30 min, 56%; b) LiOH, THF/MeOH/H_2O (4:1:1), 72 min, 99%; c) DCC/HOBt, THF, then phenylalanine methyl ester/TEA, 18 h, 70%; d) Dess-Martin reagent, CH_2Cl_2, 90 min, 73%; e) H_2/Pd/C, MeOH/H_2O/HCl, 96%.

In conclusion, the first two approaches towards the synthesis of Segment A as set out in Chapter 2.3.2 were proven to be nonviable, since the unprotected guanidino group (pathway 1, Scheme 2.7) as well as the (bis-Z)-guanidino group (pathway 2) were evidently not compatible with the synthetic operations necessary for successful elaboration of α-hydroxy-β-homoarginine **58** into the target compound. This implied that the Z groups were to be exchanged (pathway 3), preferentially for protecting groups that are more acid-labile [Section 3.3]. The Boc and 1-adamantoxycarbonyl (Adoc) group, removable by brief treatment with TFA, were expected to be the best candidates.

3.3. Synthesis of N^{β}-Teoc, $N^{\varepsilon,\omega}$-(bis-Adoc)-α-Hydroxy-β-Homoarginine: Exchange of Protecting Groups

Before replacing the Z groups in **58**, protection of the β-amino group was necessary. This new protecting group for the β-amine should be:

(1) resistant to catalytic hydrogenation (as the Z groups were to be removed by hydrogenation),

(2) not sensitive to bases, base-treatment was foreseen to achieve ester hydrolysis,

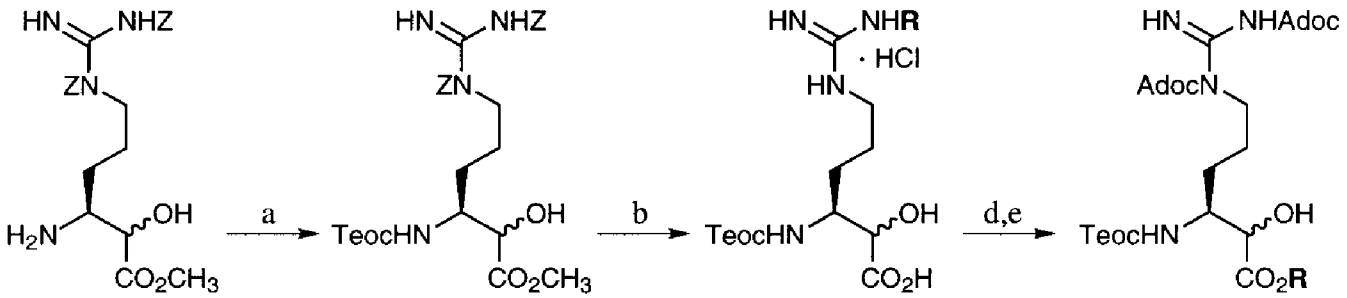
(3) orthogonal to the acid-labile guanidine protective groups, and

(4) not susceptible to nucleophilic attack by the α-hydroxyl group.

The (2-trimethylsilyl)ethoxycarbonyl (Teoc) group,[25] removable by fluoride-induced β-elimination, meets these requirements. Thus, **58** was reacted with 2-(trimethylsilyl)ethyl 4-nitrophenyl carbonate, prepared from 2-(trimethylsilyl)ethanol,[25a,26] to give the fully protected α-hydroxy-β-homoarginine methyl ester **69** in 43 % yield [Scheme 3.11].

Treatment with LiOH removed both the methyl ester and, as expected now, the N^{ε}-Z group to give **70**. Subsequent catalytic hydrogenolysis produced N^{β}-Teoc-α-hydroxy-β-homoarginine (**71**).

Scheme 3.11. Exchange of the guanidine protecting groups in α-hydroxy-β-homoarginine **58**.



a) Teoc-OPnp/DMAP, MeCN, 28 h, 43%; b) 1) LiOH, THF/MeOH/H_2O (4:1:1), 75 min, 2) HClaq, 85%; c) H_2/Pd/C, MeOH/HClaq, 90%; d) Adoc-Cl/NaOHaq, dioxane/H_2O, 0 °C, 5 h; e) CH_2N_2, CH_2Cl_2/EtOH, 0 °C, 43% (two steps); f) LiOH, THF/MeOH/ H_2O (4:1:1), 75 min, 90%.

In this derivative we now could introduce the two desired acid-labile guanidine protecting groups. As will be discussed in Section 3.4.1, selective introduction of two Boc groups into a guanidino moiety of arginine is not possible; however, $N^{\delta,\omega}$-(bis-Adoc) protected arginines are readily prepared from N^{α}-protected arginine derivatives and commercially available 1-adamantyl fluoroformate (Adoc-F) under Schotten-Baumann conditions.[27]

The first attempt to introduce two Adoc groups in **71** gave a mixture of compounds with a rather complex ^{1}H-NMR spectrum. We considered that the Teoc group might have been attacked by fluoride, released by nucleophilic displacement from Adoc-F, and that the liberated amine would react with the excess (3.5 equiv) of Adoc-F used. Furthermore, it also appeared that the hydroxyl group, to some extent, was substituted with an Adoc group.

Using 1-adamantyl chloroformate (Adoc-Cl),[28] prepared from 1-adamantanol and phosgene,[29] instead of Adoc-F, the desired N^{β}-Teoc, $N^{\varepsilon,\omega}$-(bis-Adoc)-α-hydroxy-β-homoarginine (**73**) was obtained. However, **73** could not be separated from 1-adamantanol formed by hydrolysis of excess Adoc-Cl present in the crude reaction product. Therefore, the crude α-hydroxy acid was treated with diazomethane to give α-hydroxy ester **72**, which was purified by column chromatography. Subsequent saponification of the methyl esters of **72** with LiOH gave pure **73** in 90% yield.
As both Adoc groups were retained under these basic conditions, this hydrolysis shows nicely the higher stability of the N^{ε}-Adoc group compared to the N^{ε}-Z group.

In summary, the sequence **46** → **58** [29%, Scheme 3.6], **58** → **73** [13%, Scheme 3.11] yielded α-hydroxy-β-homoarginine **73** with the desired orthogonal protecting groups, including two acid-labile groups in the arginine side chain. Although the overall yield of this laborious reaction sequence (**46** → **73**) was rather low (4%), we prepared enough of **73** to study the synthesis of Segment A [Chapter Four].

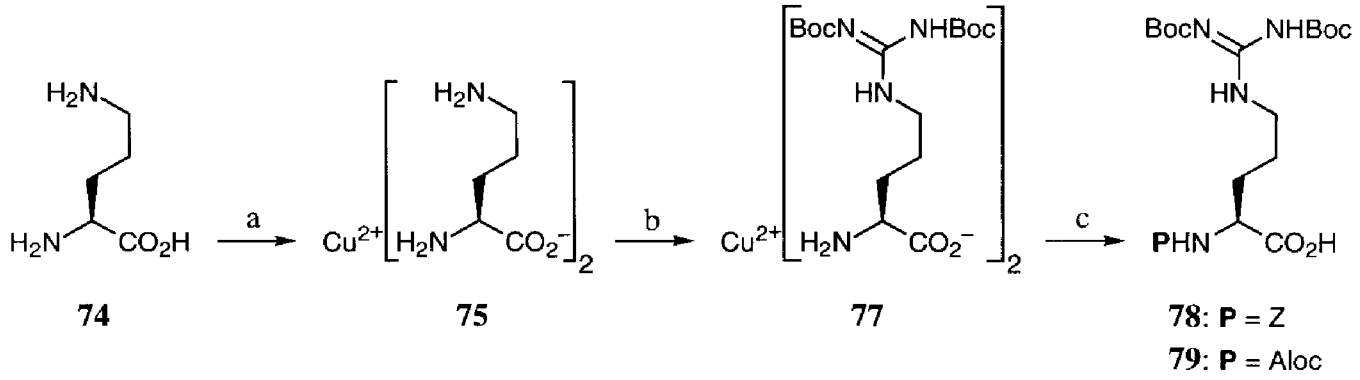
3.4. Synthesis and Homologation of N^{α}-Z, $N^{\omega,\omega'}$-(bis-Boc)Arginine: A New Protecting Group Strategy

In Section 3.3 the necessity of acid-labile protecting groups for the guanidine moiety of the α-hydroxy-β-homoarginine building block was established. Thus, we were forced to exchange the Z groups of **58** for Adoc groups before we could focus on further elaboration of this building block.
However, we realized that it would be far more efficient and elegant to develop a route to an α-hydroxy-β-homoarginine derivative with acid-labile guanidine protection, starting with an arginine derivative containing already such acid-labile protecting groups.
In this section the preparation of two arginine derivatives with a bis-Boc protected guanidino group, *i.e.* N^{α}-Z, $N^{\omega,\omega'}$-(bis-Boc)arginine (**78**) and N^{α}-Aloc, $N^{\omega,\omega'}$-(bis-Boc)arginine (**79**) [Scheme 3.12], and their successful use in the tris(methylthio)methane based homologation sequence is discussed.

3.4.1. Preparation of $N^{\omega,\omega'}$-(bis-Boc)arginine derivatives

Direct acylation of N^{α}-Z-arginine with di-*tert*-butyl dicarbonate (Boc_2O) or Boc-azide produced a mixture of $N^{\delta,\omega}$-(bis-Boc)arginine and $N^{\omega,\omega'}$-(bis-Boc)arginine derivatives. This observation had also been made by other researchers and inspired them to devise a synthesis of unambiguously protected (bis-Boc)arginine derivatives.[30]
In two recent papers on the synthesis of N^{α}-Fmoc, $N^{\omega,\omega'}$-(bis-Boc)arginine, the reagents bis-Boc-*S*-methylisothiourea[30,31] and N^{1}-(*N,N'*-(bis-Boc)amidino)pyrazole[32] are used to convert ornithine to the desired $N^{\omega,\omega'}$-(bis-Boc)arginine derivative. Synthesis *via* the pyrazole based reagent is more convenient because it utilises Cu(II)-ornithine complex **75** [Scheme 3.12] instead of a N^{α}-protected ornithine derivative, which has to be prepared by a four step synthesis using the same Cu(II)-ornithine complex. Thus, Cu(II)-ornithine complex **75**[33] was first converted to arginine derivative **77** with N^{1}-(*N,N'*-(bis-Boc)amidino)pyrazole[32] and subsequently reacted with Z-Cl/EDTA/$NaHCO_3$ to furnish **78**.
In addition, a second orthogonally protected arginine derivative, *i.e.* **79** with a N^{α}-Aloc, group was prepared analogously.

Scheme 3.12. Preparation of $N^{\omega,\omega'}$-(bis-Boc)arginine derivatives starting from ornithine.



74 **75** **77** **78: P = Z** **79: P = Aloc**

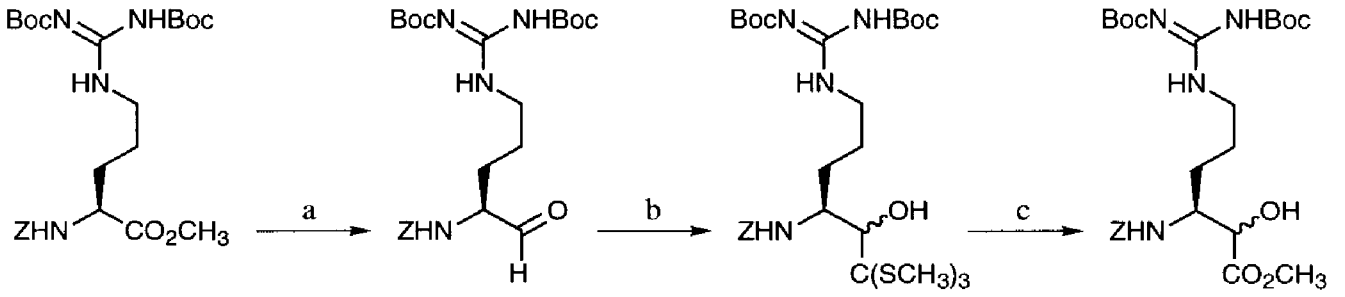
a) $CuCO_3$, H_2O, 100 °C, 1 h, 87%; b) N^1-(*N,N'*-(bis-Boc)amidino)pyrazole/DiPEA, formamide, 24 h; 90% c) **78**: Z-Cl/EDTA/$NaHCO_3$, H_2O/acetone, 12 h, 60%, **79**: Aloc-Cl/EDTA//$NaHCO_3$, H_2O/acetone, 12 h, 55%.

3.4.2. Homologation of $N^{\omega,\omega'}$-(bis-Boc)arginine derivatives 78 and 79

We realized that for the homologation of **78** or **79** Route II [Scheme 3.2] could not be used, as the two Boc groups would not survive the acidic conditions required for hydrolysis of the cyanohydrin intermediate [Section 3.2.2]. Route I might be more successful this time, as we anticipated that in the aldehyde derivatives from **78** and **79** de-amidation was less likely to occur (as compared to **47**, Scheme 3.4) due to the decreased electrophilic nature of the imino carbon atom, the decreased leaving group character of the N^{δ}, and the increased bulkiness of the protecting groups.

So, methyl ester **80**, prepared from acid **78** by treatment with diazomethane [Scheme 3.13], was reduced to aldehyde **81** under the same conditions as used for the preparation of **47** [Scheme 3.3]. Addition of tris(methylthio)methyllithium to the crude aldehyde at –65 °C gave α-hydroxy ortho-thioester **82**; indeed, no de-amidation was observed. Treatment of this orthothioester with $HgCl_2$/HgO in MeOH/H_2O (92:8) at room temperature for 64 h afforded α-hydroxy-β-homoarginine derivative **83** (mixture of diastereomers, 87:13) in an overall yield of 41% from methyl ester **80**.

Scheme 3.13. Synthesis of α-hydroxy-β-homoarginine derivative **83** via Route I.



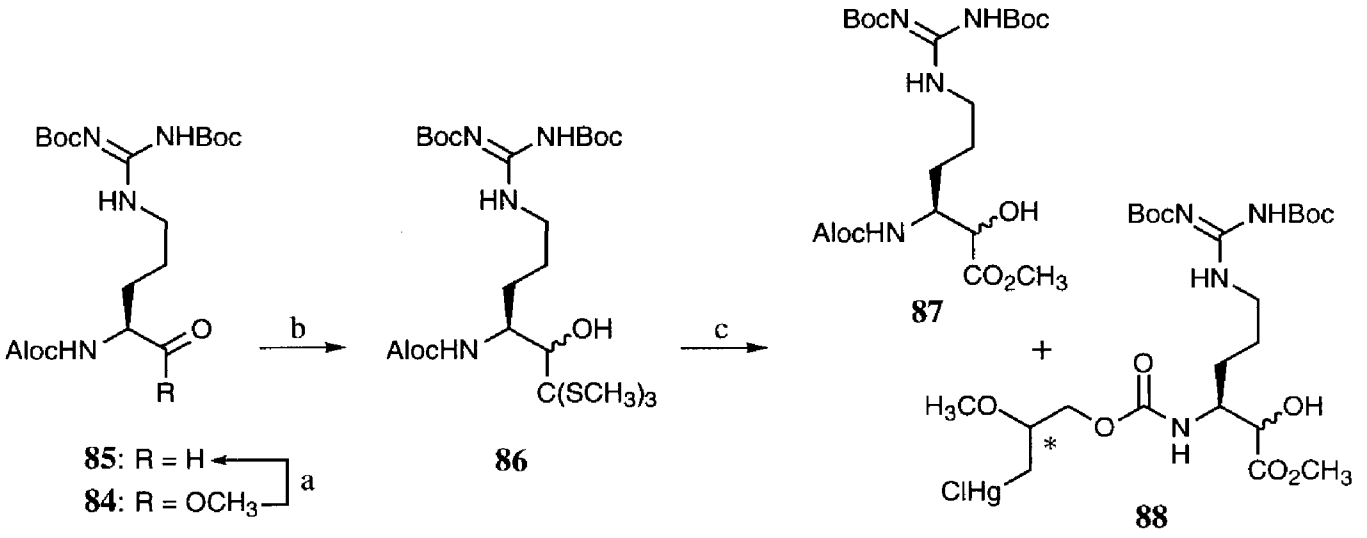
80 **81** **82** **83**

a) DiBAH, CH_2Cl_2, –65 °C, 75 min, 97%; b) $LiC(SCH_3)_3$, THF, –65 °C, 5 h, 54%; c) $HgCl_2$/HgO, MeOH/H_2O (92:8), rt, 64 h, 79%.

Homologation of the second arginine derivative, *i.e.* **84** (prepared from **79** with diazomethane), was less satisfactory [Scheme 3.14]. The Aloc group was found to be unstable under the conditions for

Hg^{2+}-mediated hydrolysis of orthothioester **86** and only 15% of the desired α-hydroxy ester **87** was isolated. The major product (61%) was α-hydroxy ester **88** in which the Aloc group had been oxymercurated.[34]

Scheme 3.14. Homologation of N^{α}-Aloc, $N^{\omega,\omega'}$-(bis-Boc)arginine methyl ester (**84**).

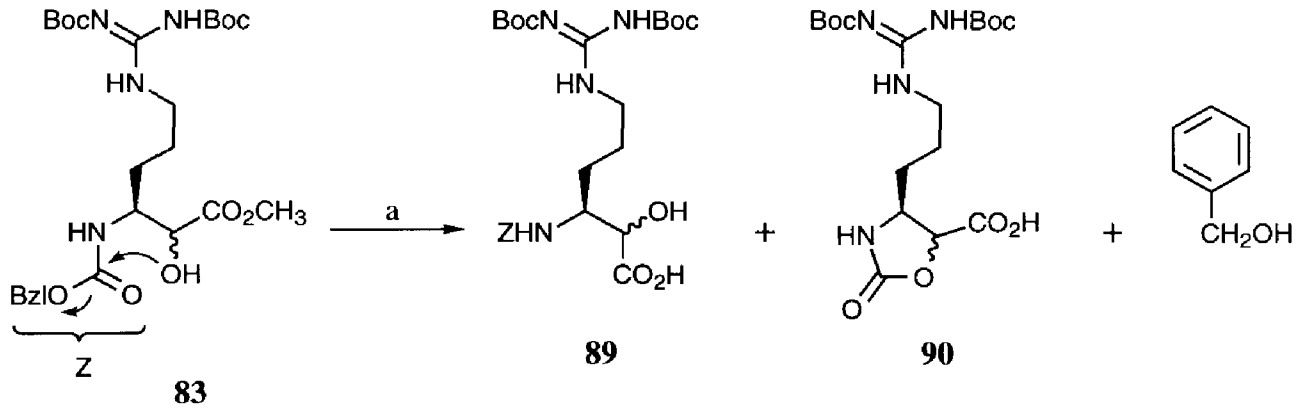


a) DiBAH, CH_2Cl_2, –65 °C, 75 min, 77%; b) $LiC(SCH_3)_3$, THF, –65 °C, 5 h, 72%; c) $HgCl_2/HgO$, $MeOH/H_2O$ (92:8), rt, 64 h, **87**: 15%, **88**: 61%.

3.4.3. Selective deprotection of N^{β}-Z, $N^{\omega,\omega'}$-(bis-Boc)-α-hydroxy-β-homoarginine methyl ester (83)

Upon hydrolysis of methyl ester **83**, under standard conditions [LiOH 3 equiv, THF/MeOH/H_2O (4:1:1), rt, 72 min] acid **89** was isolated in addition to a considerable amount of cyclic carbamate **90** [Scheme 3.15]. This is probably due to an intramolecular nucleophilic attack by the hydroxyl group at the carbonyl group of the Z group followed by displacement of benzyl alcohol, in an analogous fashion as was observed for dipeptide **59** [Scheme 3.9]. Also Maryanoff *et al.* have reported the formation of a cyclic carbamate during the saponification of N^{β}-Z, N^{ω}-Ts-α-hydroxy-β-homoarginine.[35]

Scheme 3.15. Formation of oxazolidinone **90** upon hydrolysis of α-hydroxy-β-homoarginine **83**.



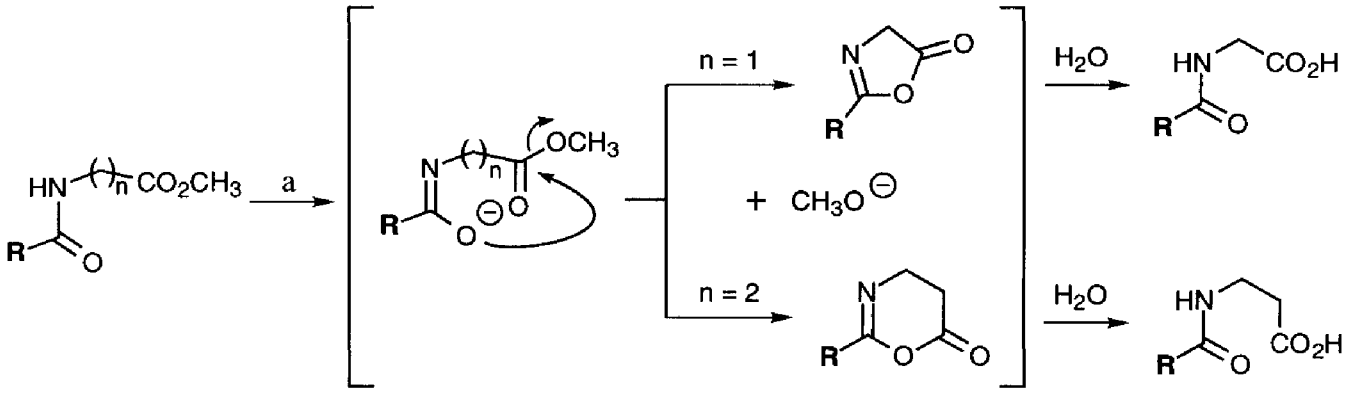
a) LiOH, THF/MeOH/H_2O (4:1:1), rt, 72 min, **89**: 65%, **90**: 35%.

Despite the usefulness of oxazolidinone **90** in the elucidation of the stereochemistry of α-hydroxy-β-homoarginine derivative **83** (*vide infra*), its formation decreased the yield of the desired acid **89** significantly.

In an effort to prevent oxazolidinone formation we considered using an Aloc group instead of a N^{β}-Z group since the Aloc group was found to be stable during the hydrolysis of a N^{β}-Aloc protected α-hydroxy-β-amino ester [Section 3.2.3]. However, homologation of N^{α}-Aloc arginine derivative **79** was unsuccessful [Section 3.4.2]. As protection of the hydroxyl group in **83** is likely to prevent oxazolidinone formation we focused again on the introduction of a hydroxyl protecting group. Earlier we had already observed that protection of the hydroxyl group as a silyl ether prevented saponification of the methyl ester [Section 3.2.3]. We now found that attempts to form a tetrahydropyranyl or an ethoxyethyl ether were unsuccessful. Therefore, we decided to investigated the rate of ester hydrolysis of in **83** by LiOH. It was surprising and gratifying to find that after only 12 min the ^{1}H-NMR signal of the methyl ester had completely disappeared, and that no signals due to oxazolidinone formation had appeared yet. Hence, decreasing the reaction time from 72 to 12 min prevented the formation of detectable amounts of oxazolidinone and gave acid **89** as the only product, in almost quantitative yield.

Probably, the high rate of methyl ester hydrolysis is caused by a neighbouring group effect, as a control experiment with *n*-nonanoic acid methyl ester showed only 50% hydrolysis after 12 min, and 90% after 72 min. Not only α-hydroxy-β-amino ester derivatives exhibit this fast hydrolysis, also simple α-amino acid methyl esters (*N* protected or at the *C*-terminus of a peptide) gave the corresponding acid quantitatively after 12 min. However, γ-amino acid methyl esters showed essentially the same reactivity as *n*-nonanoic acid methyl ester. Apparently, a carbonyl group at an α- or β-amine promotes hydrolysis of the methyl ester, possibly through the intermediacy of a 5- or 6-membered ring [Scheme 3.17].

Scheme 3.17. Neighbouring group effect in the hydrolysis of α-amino and β-amino acid methyl esters.



a) LiOH, THF/MeOH/H_2O (4:1:1), rt, 12 min. **R** = OBzl, O-*t*-Bu, or an amino acid residue.

In conclusion, we succeeded in the straightforward preparation of α-hydroxy-β-homoarginine derivative **89** with acid-labile guanidine protecting groups. The route to **89** (8 steps from ornithine *i.e.* **74**, 19%) is far more efficient than the synthesis of N^{β}-Teoc, $N^{\delta,\omega}$-(bis-Adoc)-α-hydroxy-homoarginine (**73**) *via* the cyanohydrin based route followed by exchange of protecting groups (12 steps from arginine (**30**), 4%). The preparation of Segment A starting with **89** is described in Chapter Four.

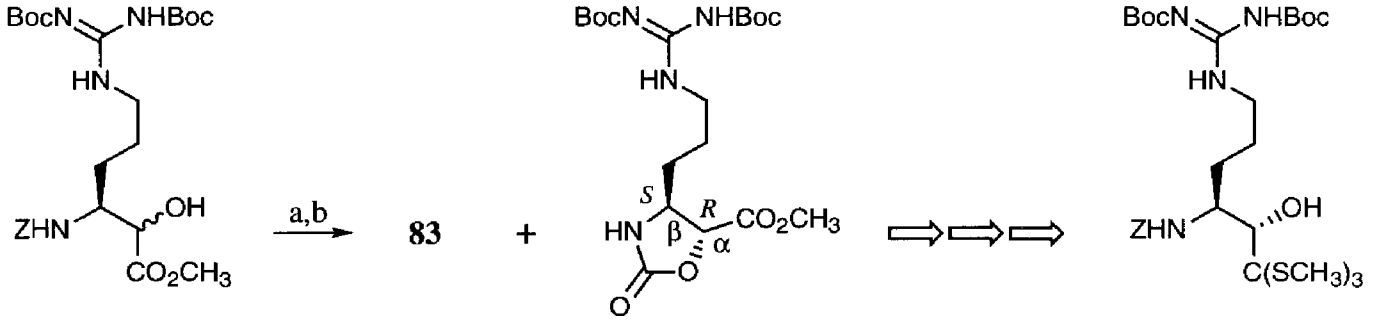
3.4.4. Elucidation of the stereochemistry of α-hydroxy-β-homoarginine derivatives

It was realized that the isolation and characterization of oxazolidinone **90** might allow assignment of the stereochemistry of the C_α-atom of **83**.[36] This was of interest as α-hydroxy amides are known to exhibit potent enzyme inhibiting activity. Therefore, a Cyclotheonamide derivative with an α-hydroxy amide unit instead of an α-keto amide might be an interesting analogue and worthwhile to prepare [Chapter Seven]. However, the biological activity of α-hydroxy acids derivatives is often strongly dependent on the stereochemistry of the C_α-atom, so separation of the epimers and assignment of their absolute stereochemistry is required.

Due to the very characteristic ^{1}H- and ^{13}C-NMR data of **90**, in particular the ^3J(HH)-coupling constants, elucidation of their stereochemistry is quite straightforward.[36] In the ^{1}H-NMR, the signal of the H_α-atom of a *cis* oxazolidinone is expected to appear down field ($\delta \approx 5$ ppm) and to have a larger coupling constant ($J \approx 8$ Hz) when compared to the *trans* isomer ($\delta \approx 4.5$ ppm, $J \approx 5$ Hz).

A sample of a reaction mixture, obtained by hydrolysis of **83**, containing oxazolidinone **90** and α-hydroxy-β-homoarginine **89** was treated with diazomethane to give, after chromatography, methyl oxazolidinonecarboxylate **91a** and methyl ester **83**, in 28% and 66% yield, respectively [Scheme 3.16]. The C_α-epimer of **91a** was not observed. Perusal of the ^{1}H-NMR spectrum of **91a** gave a $^3J(H_\alpha H_\beta)$-coupling constant of 4.6 Hz ($\delta_{H\alpha} = 4.56$ ppm) indicating a *trans* relationship of these H-atoms, and, therefore the *R*-configuration for the C_α-atom of **91a**. Since *trans* oxazolidinone **91a** corresponds to the major isomer formed upon addition of tris(methylthio)methyllithium to *S*-aldehyde **81**, we assigned the (1*R*, 2*S*) configuration to the major isomer (*i.e.* **82a**). This is in agreement with the model of Cram which predicts also the (1*R*, 2*S*) configuration for the product of addition of tris(methylthio)-methyllithium to aldehyde **81** (S = H, M = NHZ, and L = arginine side chain).

Scheme 3.16. Elucidation of the stereochemistry of α-hydroxy-β-homoarginine derivatives.



83 (83:17) (85:15) **91a** **82a**: major isomer

a) LiOH, THF/MeOH/H_2O (4:1:1), rt, 72 min; b) CH_2N_2, CH_2Cl_2, 0 °C, **83**: 66%, **91a**: 28% (two steps).

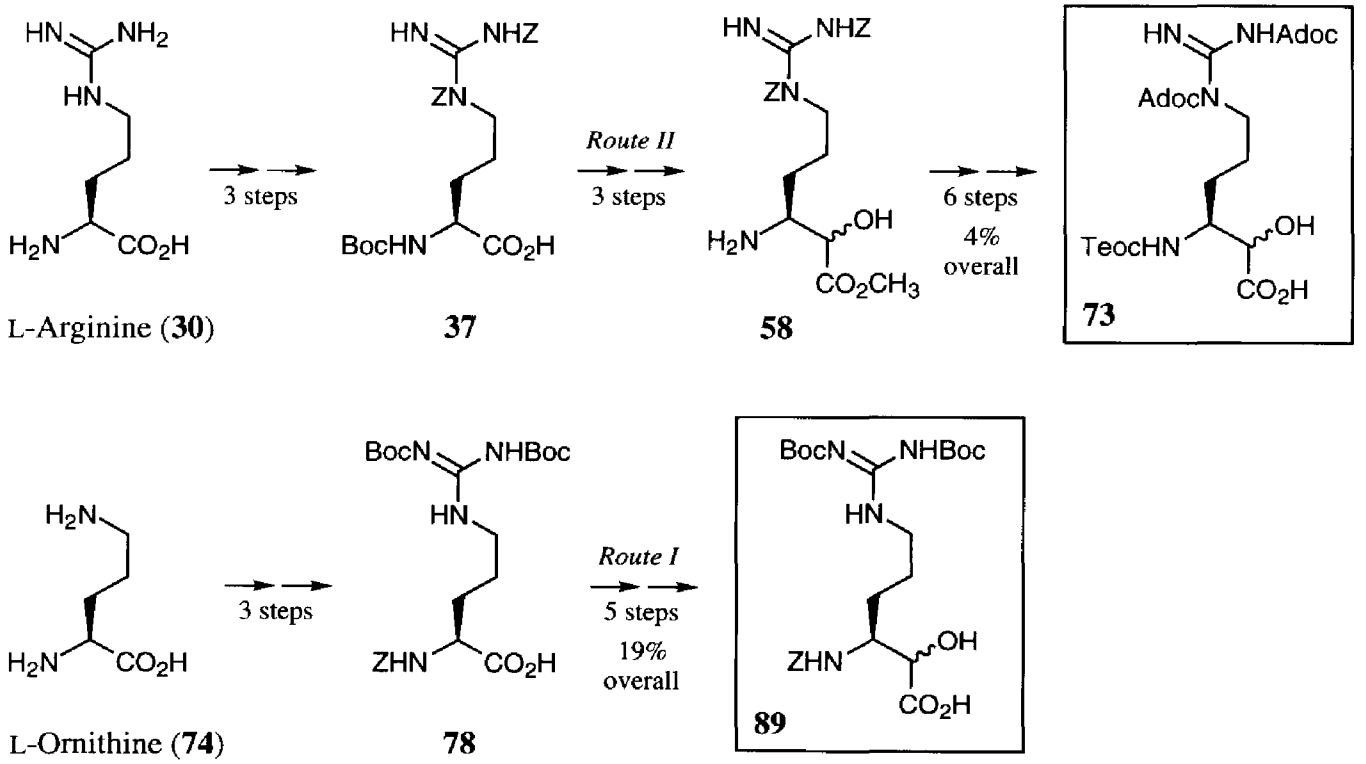
3.5 Summary and Conclusions

Proper protection of the arginine unit, especially of the guanidino group, was found to be of paramount importance, not only during homologation, but certainly also during further elaboration of the α-hydroxy-β-homoarginine building block. Our first approach towards a properly protected α-hydroxy-

β-homoarginine derivative started with arginine, which was converted into $N^{\delta,\omega}$-(bis-Z) protected **37** [Scheme 3.18]. It became apparent, however, that the bis-Z protecting group strategy was not optimal for the adequate protection of arginine's guanidino unit. The bis-Z protected guanidino group was found to be labile during homologation *via* Route I, as well as *via* Route II (Scheme 3.2). Strong nucleophiles such as tris(methylthio)methyllithium, required for Route I, caused de-amidation, and under conditions for cyanohydrin formation (Route II) one of the Z groups was cleaved. However, under carefully controlled conditions, homologation of **37** *via* Route II eventually gave the desired α-hydroxy-β-homoarginine derivative **58**.

In a set of model reactions, using α-hydroxy-β-homoarginine derivatives, and selected to establish the optimal pathway towards the target compound, it was found that the presence of at least one Z group on the guanidino function was necessary to enable oxidation of an α-hydroxy-β-homoarginine to an α-oxo-β-homoarginine derivative. Unfortunately, acid-catalyzed removal of the Z group of an α-oxo-β-homoarginine dipeptide was accompanied by extensive degradation of the product. Hence, it was concluded that the Z groups in β-homoarginine **58** were to be exchanged for protecting groups that are more acid-labile (Adoc), to facilitate oxidation of the hydroxyl group and subsequent mild guanidine deprotection in a late-stage of the synthesis. The resulting orthogonally protected α-hydroxy-β-homoarginine derivative **73** is a useful building block in the synthesis of Segment A.
Moreover, a second approach, using a different protecting group strategy was developed. α-Hydroxy-β-homoarginine derivative **89**, was prepared, more efficiently now, from L-ornithine (**74**) *via* arginine derivative **78** which already contains acid-labile guanidine protecting groups. Compound **78** was successfully homologated *via* Route I, using tris(methylthio)methyllithium, to give **89**. The latter compound would prove to be an excellent building block for the synthesis of Segment A [Chapter Four].

Scheme 3.18. Synthesis α-hydroxy-β-homoarginine derivatives with acid-labile guanidine protection.



3.6 Experimental

General information

NMR spectra were recorded on a Bruker AC 200 (^{1}H-NMR: 200.1 MHz; ^{13}C-NMR; 50.29 MHz) and on a Bruker MSL 400 spectrometer (^{1}H-NMR: 400.1 MHz; ^{13}C-NMR; 100.63 MHz). ^{1}H-NMR chemical shifts (δ) are reported in ppm relative to $CHCl_3$ (δ= 7.27 ppm) or DMSO-d_5 (δ= 2.50 ppm); ^{13}C-NMR chemical shifts (δ) are reported in ppm relative to $^{13}CDCl_3$ (δ= 77.0 ppm) or ^{13}C-DMSO-d_6 (δ= 39.5 ppm). Coupling constants (*J*) are given in Hz. 2D-NMR (H-H and C-H) COSY, and DEPT-techniques were frequently used to support interpretation of 1D spectra.

FAB(-HRMS) measurements were performed on a Finnigan MAT 90 spectrometer equipped with a WATV Cs ion gun, operated at a beam current of approx 2 μA at 25 KV. GC-MS analyses were performed on an HP-5980 GC/5970 MS combination operating at 70 eV and equipped with a Chrompack BP1 (QSGE) 50 m/0.25 mm column. Optical rotations were measured on a Perkin Elmer polarimeter model 241 MC. Melting points were recorded on a Kofler hot stage apparatus under a Reichert microscope and are uncorrected.

Medium pressure liquid column chromatography (MPLC) was performed on a Jobin-Yvon "Miniprep" LC apparatus with a stainless steel column (300 mm x 40 mm), using UV (254 nm) and refraction index detection. Silica gel 60H (Merck 7736) was used as stationary phase. For preparative column chromatography at atmospheric pressure silica gel 60 (Merck 7733) was used.
Centrifugal chromatography was performed on a "Chromatotron" model 8924 (Harrison Research), using silica gel 60 PF_{254} (Merck 7749) coated (4 mm) glass rotors with UV detection. Preparative thin-layer chromatography was performed on 2 mm silica gel 60 F_{254} coated glass plates (Merck 5717). Analytical thin-layer chromatography was performed on 0.2 mm silica gel 60 F_{254} coated aluminium plates (Merck 5554). Spots were visualised using UV detection, iodine vapour, pyrolysis or ninhydrin.

Dry solvents used: THF distilled from NaH and, subsequently, from sodium benzophenone ketyl; Et_2O distilled from NaH; CH_2Cl_2 and 1,2-dichloroethane dried over molecular sieves (4Å); MeOH dried over molecular sieves (3Å). Glassware was usually oven-dried overnight at 140 °C, assembled hot and flushed with nitrogen before use. All reactions were performed under a nitrogen atmosphere, unless stated otherwise.

Methyl 2(*S*)-[(*tert*-butyloxycarbonyl)amino]-5-[[imino[(phenylmethoxycarbonyl)amino]-methyl](phenylmethoxycarbonyl)amino]pentanoate (46)

A stirred solution of N^{α}-Boc, $N^{\delta,\omega}$-(bis-Z)arginine[13] (**32**) (10.2 g, 18.8 mmol) in CH_2Cl_2 (130 mL) containing EtOH (4 mL) was cooled in an ice-bath while diazomethane, generated from Diazald® or Diazogen® in an apparatus described by Lombardi,[14] was bubbled through until a persistent yellow colour was observed. The ice-bath was removed and the reaction mixture was flushed with nitrogen to remove the excess of diazomethane. After drying (Na_2SO_4), the reaction mixture was filtrated, concentrated under reduced pressure, and crystallized from isopropyl alcohol to give **46** as a colourless amorphous solid (10.0 g; 95,6%); mp 80.5-81 °C.
^{1}H-NMR ($CDCl_3$): 1.40 (s, 9H, Boc), 1.50-1.82 (m, 4H, β- and γ-H), 3.61 (s, 3H, OCH_3), 3.95 (t,

J=6.7, 2H, δ-H), 4.29 (m, 1H, α-H), 5.09 (d, *J*=8.6, α-NH), 5.12 (s, 2H, OC$\underline{H}_2$Ph), 5.21 (s, 2H, OC$\underline{H}_2$Ph), 7.25-7.45 (m, 10H, aryl), 9.25 and 9.50 (2x bs, 2H, ω- and ω'-NH). ^{13}C-NMR ($CDCl_3$): 24.7 (γ), 28.1 (C($\underline{C}H_3)_3$), 29.2 (β), 44.0 (δ), 52.0 (OCH_3), 54.3 (α), 66.8 (O$\underline{C}H_2$Ph), 68.7 (O$\underline{C}H_2$Ph), 79.8 (O$\underline{C}(CH_3)_3$), 127.6 (aryl-4), 128.2 (aryl-3,5), 128.7 (aryl-2,6), 134.5 (aryl-1), 136.8 (aryl-1), 155.3 (C(O), Boc), 155.6 (C(O)O, Z), 160.3 (C=N), 163.7 (C(O)O, Z) and 172.9 ($\underline{C}$(O)OCH_3).

2(*S*)-[(*tert*-Butoxycarbonyl)amino]-5-[[imino[(phenylmethoxycarbonyl)amino]methyl]-(phenylmethoxycarbonyl)amino]pentanal (47)

To a stirred solution of methyl ester **46** (13.0 g, 23.4 mmol) in CH_2Cl_2 (234 mL) at –65 °C was added DiBAH (1 N in hexanes, 81.8 mL, 81.8 mmol) at such rate as to keep the temperature below –63 °C (*ca* 45 min). After the reaction was complete (usually after an additional 30-45 min, monitored by TLC) a solution of EtOH/36% HCl_{aq} (4.7 mL, 9:1) was added slowly (the temperature was kept below –63 °C). The cold reaction mixture was then added to a vigorously stirred solution of HCl_{aq} (1 N, 700 mL) at 0 °C, stirred for 3 min, and the layers were separated. The aqueous phase was extracted with ice-cold CH_2Cl_2 (2x 250 mL), and the combined organic extracts were washed, sequentially, with ice-cold HCl_{aq} (1 N, 200 mL), ice-cold H_2O (3x 300 mL) and ice-cold brine (200 mL), dried (Na_2SO_4), filtrated and concentrated under reduced pressure, to give **47** as colourless oil (11.3 g; 91.9% crude yield). Usually, the CH_2Cl_2 solution of the crude aldehyde was not concentrated but immediately used in the cyanohydrin synthesis.

^{1}H-NMR ($CDCl_3$): 1.42 (s, 9H, Boc), 1.59-1.84 (m, 4H, β- and γ-H), 3.87 (m, 2H, δ-H), 4.11 (m, 1H, α-H), 5.11 (s, 2H, OC$\underline{H}_2$Ph), 5.22 (s, 2H, OC$\underline{H}_2$Ph), 5.48 (d, *J*=8.0, α-NH), 7.25-7.45 (m, 10H, aryl), 9.27 and 9.42 (2x bs, 2H, ω- and ω'-NH) and 9.42 (s, 1H, C(O)H). ^{13}C-NMR ($CDCl_3$): 24.4 (γ), 25.3 (β), 28.1 (C($\underline{C}H_3)_3$), 44.0 (δ), 59.1 (α), 66.8 (O$\underline{C}H_2$Ph), 68.9 (O$\underline{C}H_2$Ph), 79.8 (O$\underline{C}(CH_3)_3$), 127.7 (aryl-4), 128.3 (aryl-3,5), 128.8 (aryl-2,6), 134.4 (aryl-1), 136.6 (aryl-1), 155.6 (C(O)O, Z), 155.6 (C(O)O, Boc), 160.4 (C=N), 163.5 (C(O)O, Z) and 200.1 (C(O)H).

Methyl 2(*S*)-[(phenylmethoxycarbonyl)amino]-5-[[imino[(phenylmethoxycarbonyl)amino]-methyl](phenylmethoxycarbonyl)amino]pentanoate (48)

Prepared from $N^{\alpha,\delta,\omega}$-(tris-Z)arginine[13] (7.38 g, 12.8 mmol) following the same procedure as described for the synthesis of **46**. The crude product was purified by column chromatography (CH_2Cl_2/EtOAc, 9:1) to give ester **48** as a white foam (6.56 g; 86.7%); mp 106.5-108 °C.

^{1}H-NMR ($CDCl_3$): 1.53-1.91 (m, 4H, β- and γ-H), 3.61 (s, 3H, OCH_3), 3.95 (m, 2H, δ-H), 4.29-4.43 (m, 1H, α-H), 5.07 (s, 2H, OC$\underline{H}_2$Ph), 5.11 (s, 2H, OC$\underline{H}_2$Ph), 5.20 (s, 2H, OC$\underline{H}_2$Ph), 5.50 (d, *J*=8.6, α-NH), 7.23-7.42 (m, 15H, aryl), 9.25 and 9.43 (2x bs, 2H, ω- and ω'-NH). ^{13}C-NMR ($CDCl_3$): 24.6 (γ), 28.6 (β), 44.0 (δ), 52.2 (OCH_3), 53.8 (α), 66.9 (O$\underline{C}H_2$Ph), 66.9 (O$\underline{C}H_2$Ph), 68.8 (O$\underline{C}H_2$Ph), 127.6 (aryl-4), 128.2 (aryl-3,5), 128.7 (aryl-2,6), 134.5 (aryl-1), 136.0 (aryl-1), 136.6 (aryl-1), 155.6 (C(O)O, Z), 156.2 (C(O)O, Z), 160.3 (C=N), 163.4 (C(O)O, Z) and 172.6 ($\underline{C}$(O)OCH_3).

2(*S*)-[(Phenylmethoxycarbonyl)amino]-5-[[imino[(phenylmethoxycarbonyl)amino]methyl]-(phenylmethoxycarbonyl)amino]pentanal (49)

Prepared from methyl ester **48** (11.8 g, 20.0 mmol) according to the same procedure as described for the synthesis of **47**, yielding aldehyde **49** as a colourless oil (9.48 g; 84.6% crude yield).

^{1}H-NMR ($CDCl_3$): 1.48-1.85 (m, 4H, β- and γ-H), 3.95 (m, 2H, δ-H), 4.22 (m, 1H, α-H), 5.10 (s, 2H, OC$\underline{H}_2$Ph), 5.17 (s, 2H, OC$\underline{H}_2$Ph), 5.27 (s, 2H, OC$\underline{H}_2$Ph), 5.73 (d, *J*=8.0, α-NH), 7.23-7.42 (m, 15H, aryl), 9.25 and 9.42 (2x bs, 2H, ω- and ω'-NH) and 9.50 (s, 1H, C(O)H).

2(*R,S*)-Acetoxy-3(*S*)-[(phenylmethoxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)-amino]methyl](phenylmethoxycarbonyl)amino]hexanenitril (50)

To solution of crude aldehyde **49** (9.45 g, max 16.9 mmol) in CH_2Cl_2 (650 mL) was added H_2O (325 mL). The vigorously stirred two-phase system was placed in an ice-bath, and NaCN (8.45 g, 0.169 mol) was added. After 3 min, acetic anhydride (4.04 mL, 42.8 mmol) was added, followed by tetra-butylammonium bromide (1.41 g, 4.43 mmol). After stirring for 45 min, the reaction was complete (monitored by TLC). The aqueous phase was separated and extracted with CH_2Cl_2 (3x 150 mL). The combined organic extracts were washed thoroughly with water and brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo*. The residue, a yellow oil, was purified by column chromatography (CH_2Cl_2/EtOAc, 92:8) to give cyanohydrin **50** as a white foam (diastereomers (13:5), 8.60 g; 68.3%, based on ester **48**).

^{1}H-NMR ($CDCl_3$): 1.50-1.83 (m, 4H, γ- and δ-H), 1.97 and 1.99 (2x s, 3H, C(O)CH_3, diast), 3.75-4.20 (m, 3H, β-H and ε-H), 5.03 (bs, 2H, OC$\underline{H}_2$Ph), 5.09 (s, 2H, OC$\underline{H}_2$Ph), 5.27 (s, 2H, OC$\underline{H}_2$Ph), 5.37 and 5.41 (2x d, *J*=4.3, 1H, α-H, diast), 5.94 (bd, *J*=8.6 Hz, β-NH), 7.26-7.42 (m, 15H, aryl), 9.28 and 9.46 (2x bs, 1H, ω- and ω'-NH).

Methyl 2(*R,S*)-hydroxy-3(*S*)-[(phenylmethoxycarbonyl)amino]-6-[[imino[(phenylmethoxy-carbonyl)amino]methyl](phenylmethoxycarbonyl)amino]hexanoate (51)

A stirred solution of cyanohydrin **50** (15.0 g, 23.8 mmol) in Et_2O/MeOH (676 mL, 20:7) at –65 °C was treated with gaseous HCl (72.5 g, 1.99 mol) at such rate as to maintain the temperature below –50 °C. After 24 h at 4 °C the reaction mixture was cooled to –20 °C, H_2O/MeOH (210 mL, 3:1) was slowly added, and the solution was stirred at 4 °C for 1 h. Subsequently, H_2O (175 mL) was added at 4 °C, and the mixture was neutralized with solid $NaHCO_3$ (170 g, 2.00 mol) at ambient temperature. EtOAc (200 mL) was added, the layers were separated, and the aqueous phase was extracted with EtOAc (2x 200 mL). The combined organic solutions were washed with brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo* to give, after column chromatography (CH_2Cl_2/EtOAc, 4:1), **51** as a white foam (diastereomers (4:1), 7.16 g; 48.5%).

^{1}H-NMR ($CDCl_3$): 1.14-1.79 (m, 4H, γ- and δ-H), 3.04 and 3.09 (2x d, *J*=5.4, 1H, OH, diast), 3.62 and 3.69 (2x s, 3H, OCH_3, diast), 3.80-4.11 (m, 3H, β-H and ε-H), 4.18 and 4.27 (2x m, 1H, α-H, diast), 5.00 and 5.03 (2x s, 2H, OC$\underline{H}_2$Ph, diast), 5.10 (s, 2H, OC$\underline{H}_2$Ph), 5.19 and 5.20 (2x s, 2H, OC$\underline{H}_2$Ph, diast), 5.13 and 5.39 (2x bd, *J*=8.7, 1H, α-NH, diast), 7.14-7.44 (m, 15H, aryl), 9.25 and 9.40 (2x bs, 1H, ω- and ω'-NH). ^{13}C-NMR ($CDCl_3$): 25.1 and 25.5 (δ, diast), 28.4 (γ), 44.2 (ε), 52.6 (OCH_3), 53.0 and 53.5 (β, diast), 66.5 and 66.7 (O$\underline{C}H_2$Ph, diast), 66.8 (O$\underline{C}H_2$Ph), 68.8 (O$\underline{C}H_2$Ph), 71.7 and 72.4 (α, diast), 127.7 (aryl), 127.8 (aryl), 127.9 (aryl), 128.0 (aryl), 128.2 (aryl), 128.3 (aryl), 128.6 (aryl), 128.7 (aryl), 134.5 (aryl-1), 136.2 (aryl-1), 136.7 (aryl-1), 155.7 (C(O)O, Z), 155.9 and 156.3 (C(O)O, Z, diast), 160.4 (C=N), 163.6 (C(O)O, Z), 172.9 and 173.7 ($\underline{C}$(O)OCH_3, diast).

Methyl 2(*S*)-amino-5-[[imino[(phenylmethoxycarbonyl)amino]methyl](phenylmethoxy-carbonyl)amino]pentanoate (52)

To methyl ester **46** (11.3 g, 20.3 mmol) was added TFA/H_2O (30 mL, 9:1). The solution was stirred for 45 min. The volatiles were removed under reduced pressure, the sticky residue was triturated with Et_2O, and evaporated to dryness under reduced pressure, to yield **52**, essentially quantitatively, as a slightly coloured oil (17.9 g, 20.3 mmol + 75.6 mmol TFA).

^{1}H-NMR (DMSO-d_6): 1.51-1.87 (m, 4H, β- and γ-H), 3.62 (s, 3H, OCH_3), 3.88 (m, 2H, δ-H),

4.00-4.18 (m, 1H, α-H), 5.06 (s, 2H, $OC\underline{H}_2Ph$), 5.22 (s, 2H, $OC\underline{H}_2Ph$), 7.30-7.48 (m, 12H, aryl, ω-NH) and 8.40 (bs, 3H, NH_3^+).

Methyl 2(*S*)-[(allyloxycarbonyl)amino]-5-[[imino[(phenylmethoxycarbonyl)amino]methyl]-(phenylmethoxycarbonyl)amino]pentanoate (53)

To a stirred solution of **52** (17.9 g, 20.3 mmol + 75.6 mmol TFA) in MeCN (150 mL) at 0 °C was added DiPEA (20.4 mL, 118.0 mmol). Subsequently, Aloc-Cl (4.89 g, 40.6 mmol) was introduced via a syringe in 5 min. The cooling-bath was removed and the reaction mixture was stirred for 24 h. After adjusting the pH to 7 (with 1 N HCl_{aq}), MeCN was removed under reduced pressure. The residue was dissolved in EtOAc and washed, sequentially, with H_2O (2x), HCl_{aq} (1 N, 2x), H_2O (2x) and brine, decolourized with Montmarillonite K-10, dried (Na_2SO_4), filtrated and evaporated to dryness under reduced pressure. The oily residue was crystallized from isopropyl alcohol to give **53** as colourless needles (10.3 g; 93.8%); mp 89-90 °C.

^{1}H-NMR ($CDCl_3$): 1.51-1.90 (m, 4H, β- and γ-H), 3.62 (s, 3H, OCH_3), 3.97 (m, 2H, δ-H), 4.37 (m, 1H, α-H), 4.51 (d, *J*=6.7, $OC\underline{H}_2CH=CH_2$), 5.12 (s, 2H, $OC\underline{H}_2Ph$), 5.19 (dd, *J*=11.0 and 6.7, $OCH_2-CH=C\underline{H}_2$), 5.21 (s, 2H, $OC\underline{H}_2Ph$), 5.47 (d, *J*=8.6, α-NH), 5.87 (ddt, *J*=11.0 and 6.7, $OCH_2C\underline{H}=CH_2$), 7.27-7.42 (m, 10H, aryl), 9.27 and 9.41 (2x bs, 2H, ω- and ω'-NH). ^{13}C-NMR ($CDCl_3$): 24.6 (γ), 28.9 (β), 44.0 (δ), 52.2 (OCH_3), 53.5 (α), 65.6 ($O\underline{C}H_2CH=CH_2$), 66.8 ($O\underline{C}H_2Ph$), 68.8 ($O\underline{C}H_2Ph$), 117.6 ($OCH_2CH=\underline{C}H_2$), 127.7 (aryl-4), 128.2 (aryl-3,5), 128.7 (aryl-2,6), 132.5 ($OCH_2\underline{C}H=CH_2$), 134.5 (aryl-1), 136.8 (aryl-1), 155.6 (C(O)O, Aloc), 155.7 (C(O)O, Z), 160.3 (C=N), 163.7 (C(O)O, Z) and 172.5 ($\underline{C}(O)OCH_3$).

2(*S*)-[(Allyloxycarbonyl)amino]-5-[[imino[(phenylmethoxycarbonyl)amino]methyl](phenyl-methoxycarbonyl)amino]pentanal (54)

Prepared from methyl ester **53** (1.62 g, 3.00 mmol) according to the same procedure as described for the synthesis of **47** yielding aldehyde **53** as a colourless oil (1.27 g; 83.0% crude yield).

^{1}H-NMR ($CDCl_3$): 1.41-1.95 (m, 4H, β- and γ-H), 3.96 (m, 2H, δ-H), 4.21 (m, 1H, α-H), 4.55 (d, *J*=6.7, $OC\underline{H}_2CH=CH_2$), 5.15 (s, 2H, $OC\underline{H}_2Ph$), 5.23 (dd, *J*=11.0 and 6.7, $OCH_2CH=C\underline{H}_2$), 5.25 (s, 2H, $OC\underline{H}_2Ph$), 5.87 (ddt, *J*=11.0 and 6.7, $OCH_2C\underline{H}=CH_2$), 5.97 (d, *J*=8.0, α-NH), 7.27-7.45 (m, 10H, aryl), 9.28 and 9.40 (2x bs, 2H, ω- and ω'-NH) and 9.49 (s, 1H, C(O)H).

2(*R,S*)-Acetoxy-3(*S*)-[(allyloxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)amino]-methyl](phenylmethoxycarbonyl)amino]hexanenitril (55)

Prepared from crude aldehyde **54** (1.27 g, max 2.49 mmol) according to the same procedure as described for the synthesis of **50**, yielding almost pure (by ^{1}H-NMR) cyanohydrin **55** as a yellowish oil (diastereomers (3:1), 1.25 g; 71.9%, based on ester **53**). The cyanohydrin was used without further purification.

^{1}H-NMR ($CDCl_3$): 1.49-1.81 (m, 4H, γ- and δ-H), 1.97 (s, 3H, $C(O)CH_3$), 3.88-4.19 (m, 3H, β-H, ε-H), 4.48 (d, *J*=6.7, $OC\underline{H}_2CH=CH_2$), 5.12 (s, 2H, $OC\underline{H}_2Ph$), 5.18 (m, 2H, $OCH_2CH=C\underline{H}_2$), 5.20 (s, 2H, $OC\underline{H}_2Ph$), 5.32 and 5.41 (2x d, *J*=4.3, 1H, α-H, diast), 5.87 (m, $OCH_2C\underline{H}=CH_2$), 5.94 (d, *J*=8.6, β-NH), 7.27-7.40 (m, 10H, aryl), 9.25 and 9.43 (2x bs, 2H, ω- and ω'-NH). ^{13}C-NMR ($CDCl_3$): 19.9 and 20.0 ($C(O)\underline{C}H_3$, diast), 25.0 (δ), 25.4 and 25.6 (γ, diast), 43.9 (ε), 51.9 and 52.0 (β, diast), 63.1 and 64.0 (α, diast), 65.7 and 65.8 ($O\underline{C}H_2CH=CH_2$, diast), 66.8 ($O\underline{C}H_2Ph$), 68.9 ($O\underline{C}H_2Ph$), 115.1 and 115.2 ($OCH_2CH=\underline{C}H_2$, diast), 117.5 and 117.7 (CN, diast), 127.7 (aryl-4), 127.8 (aryl-4), 128.2 (aryl-3,5), 128.7 (aryl-2,6), 128.8 (aryl-2,6), 132.4 and 132.5 ($OCH_2\underline{C}H=CH_2$, diast), 134.4 (aryl-1), 136.6

(aryl-1), 155.5 (C(O)O, Z), 155.5 and 155.7 (C(O)O, Aloc, diast), 160.5 and 160.6 (C=N, diast), 163.5 (C(O)O, Z), 168.5 and 168.7 ($\underline{C}(O)CH_3$, diast).

Methyl 2(*R,S*)-hydroxy-3(*S*)-[(allyloxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)-amino]methyl](phenylmethoxycarbonyl)amino]hexanoate (56)

Prepared from cyanohydrin **55** (13.6 g, 23.5 mmol) according to the same procedure as described for the synthesis of **51** yielding, after column chromatography (CH_2Cl_2/EtOAc, 5:1), **56** as a white foam (diastereomers (3:1), 6.48 g; 47.7%).

^{1}H-NMR ($CDCl_3$): 1.49-1.76 (m, 4H, γ- and δ-H), 3.08 and 3.12 (2x d, *J*=6.7, 1H, OH, diast), 3.68 (s, 3H, OCH_3), 3.80-4.10 (m, 3H, β-H and ε-H), 4.15 and 4.23 (2x m, 1H, α-H, diast), 4.41-4.58 (m, 2H, $OC\underline{H}_2CH=CH_2$), 5.11 (s, 2H, $OC\underline{H}_2Ph$), 5.11-5.30 (m, 3H, $OCH_2CH=C\underline{H}_2$ and β-NH), 5.22 (s, 2H, $OC\underline{H}_2Ph$), 5.72-6.00 (m, $OCH_2C\underline{H}=CH_2$), 7.22-7.46 (m, 10H, aryl), 9.25 and 9.43 (2x bs, 2H, ω- and ω'-NH). ^{13}C-NMR ($CDCl_3$): 25.1 and 25.4 (δ, diast), 28.4 (γ), 44.2 (ε), 52.5 and 52.6 (OCH_3, diast), 52.0 and 53.5 (β, diast), 65.5 and 65.6 ($O\underline{C}H_2CH=CH_2$, diast), 66.9 ($O\underline{C}H_2Ph$), 68.8 ($O\underline{C}H_2Ph$), 71.6 and 72.9 (α, diast), 117.3 and 117.5 ($OCH_2CH=\underline{C}H_2$, diast), 127.7 (aryl-4), 127.8 (aryl-4), 128.2 (aryl-3,5), 128.4 (aryl-3,5), 128.7 (aryl-2,6), 128.8 (aryl-2,6), 132.6 ($OCH_2\underline{C}H=CH_2$), 134.5 (aryl-1), 136.7 (aryl-1), 155.7 (C(O)O, Z), 155.7 and 156.1 (C(O)O, Aloc, diast), 160.4 (C=N), 163.6 (C(O)O, Z), 172.9 and 173.6 ($\underline{C}(O)OCH_3$, diast).

2(*R,S*)-Acetoxy-3(*S*)-[(*tert*-butyloxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)-amino]methyl](phenylmethoxycarbonyl)amino]hexanenitril (57)

Prepared from crude aldehyde **47** (11.3 g, max 21.5 mmol) following the same procedure as described for the synthesis of **50**, yielding, after column chromatography, cyanohydrin **57** as a thick colourless oil, which solidified slowly upon standing (diastereomers (7:1), 8.34 g; 60%, based upon ester **46**).

^{1}H-NMR ($CDCl_3$): 1.41 (s, 9H, Boc), 1.42-1.83 (m, 4H, γ- and δ-H), 2.00 and 2.02 (2x s, 3H, $C(O)CH_3$, diast), 3.75-4.17 (m, 3H, β-H and ε-H), 5.08 (m, 1H, β-NH), 5.14 (s, 2H, $OC\underline{H}_2Ph$), 5.24 (s, 2H, $OC\underline{H}_2Ph$), 5.20 (s, 2H, $OC\underline{H}_2Ph$), 5.35 and 5.43 (2x d, *J*=4.7, 1H, α-H, diast.), 7.23-7.45 (m, 10H, aryl), 9.27 and 9.45 (bs, 2H, ω- and ω'-NH). FAB-HRMS; calcd for $[C_{30}H_{37}N_5O_8 + H]^+$ 596.2720, found 596.2675.

Methyl 2(*R,S*)-hydroxy-3(S)-amino-6-[[imino[(phenylmethoxycarbonyl)amino]methyl]-(phenylmethoxycarbonyl)amino]hexanoate (58)

By deprotection of 56: To a solution of **56** (4.78 g, 8.38 mmol) in THF (64 mL) was added dimedone (8.90 g, 63.5 mmol) and tetrakis(triphenylphosphine)palladium (0.96 g, 0.83 mmol). After 30 min the reaction mixture was added to HCl_{aq} (500 mL, 0.5 N), and extracted with Et_2O (3x 200 mL). The aqueous phase was adjusted to pH≈ 10 with Na_2CO_3 and extracted with EtOAc (3x 100 mL). The combined EtOAc solutions were washed with brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo* to give **58** as a yellow solid (diastereomers (3:1), 3.69 g; 90.5%). mp 83-85 °C.

By hydrolysis of cyanohydrin 57: A stirred solution of cyanohydrin **57** (12.5 g, 21.1 mmol) in Et_2O/MeOH (690 mL, 19:6) at –65 °C was treated with gaseous HCl (75.9 g, 2.08 mol) at such rate as to maintain the temperature below –40 °C. After 24 h at 4 °C the reaction mixture was cooled to –20 °C, H_2O/MeOH (200 mL, 3:1) was added in 1 h, and the resulting solution was stirred for 45 min at 4 °C. Subsequently, H_2O (200 mL) was added at 4 °C, and the mixture was carefully added to a vigorously stirred aqueous solution of $NaHCO_3$ (84.0 g, 1.0 mol in 600 mL) at ambient temperature. The layers were separated and the aqueous phase was washed with Et_2O (250 mL). After adjusting the

pH of the aqueous phase to pH≈ 10 with solid Na_2CO_3, the resulting turbid solution was extracted with EtOAc (4x 200 mL). The combined organic solutions were washed with brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo* to give **58** as an off-white solid (diastereomers (11:2), 9.47 g; 92.2%), which was used without further purification.

^{1}H-NMR ($CDCl_3$): 1.43-1.85 (m, 4H, γ- and δ-H), 1.91 (bs, 2H, β-NH_2), 2.95-3.08 (m, 1H, α-OH), 3.68 and 3.72 (2x s, 3H, OCH_3, diast), 3.95 (m, 2H, ε-H), 4.01 and 4.09 (2x m, 1H, α-H, diast), 5.10 (s, 2H, O$\underline{CH_2}$Ph), 5.22 (s, 2H, O$\underline{CH_2}$Ph), 7.23-7.40 (m, 15H, aryl), 9.24 and 9.40 (2x bs, 2H, ω- and ω'-NH). FAB-HRMS: calcd for $[C_{24}H_{30}N_4O_7 + H]^+$ 487.2913, found 487.2201.

Methyl 2(*R,S*)-hydroxy-3(*S*)-[[[1-(*tert*-butyloxycarbonyl)pyrrolidin-2(*S*)-yl]carbonyl]amino]-6-[[imino[(phenylmethoxycarbonyl)amino]methyl](phenylmethoxycarbonyl)amino]hexanoate (59)

To a stirred solution of *N*-Boc proline (1.66 g, 7.71 mmol) in CH_2Cl_2 (69 mL) at 0 °C was added HOBt (1.65 g, 11.9 mmol) and DCC (1.65 g, 8.00 mmol). After 45 min, amine **58** (3.69 g, 7.58 mmol) in CH_2Cl_2 (25 mL) was added. After another 45 min, the ice-bath was removed, and the stirring was continued for 18 h. Subsequently, the solvent was evaporated under reduced pressure, the residue was taken up in EtOAc and filtrated. The organic solution was sequentially washed with aqueous $NaHCO_3$ (5%, 3x), aqueous $KHSO_4$ (6%, 3x), H_2O (2x) and brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo* to give **59**, after purification by column chromatography (EtOAc/CH_2Cl_2, 1:1) as a white foam (2.84 g; 66.8%).

^{1}H-NMR ($CDCl_3$): 1.44 (s, 9H, Boc), 1.50-2.15 (m, 8H, hArg β- and γ-H, Pro β- and γ-H), 3.35 (m, 2H, Pro δ- H), 3.71 (s, 3H, OCH_3), 4.01 (m, 2H, hArg δ-H), 4.12-4.33 (m, 3H, hArg α-H, β-H, OH), 5.12 (s, 2H, O$\underline{CH_2}$Ph), 5.26 (m, 2H, O$\underline{CH_2}$Ph), 7.26-7.43 (m, 10H, aryl), 9.28 and 9.48 (2x bs, 2H, hArg ω- and ω'-NH).

Methyl 2(*R,S*)-hydroxy-3(*S*)-[(*tert*-butyloxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)amino]methyl](phenylmethoxycarbonyl)amino]hexanoate (60)

To a stirred solution of amino ester **58** (8.40 g, 17.3 mmol) in DMF (55 mL) at 60 °C was added Boc_2O (4.36 g, 20.0 mmol). After 30 min, the solution was allowed to cool to room temperature and the solvent was removed *in vacuo*. The residue was partitioned between H_2O (100 mL) and CH_2Cl_2 (100 mL), the layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (3x 50 mL). The combined organic layers were washed with $KHSO_4$ (6%, 3x), H_2O (2x) and brine, dried (Na_2SO_4), filtrated, and concentrated *in vacuo*. The resulting oil was purified by column chromatography (EtOAc/PE, 3:2), to give **60** as a white foam (diastereomers (11:1), 5.67 g; 55.9%).

^{1}H-NMR ($CDCl_3$): 1.39 and 1.42 (2x s, 9H, $C(CH_3)_3$, diast), 1.48-1.78 (m, 4H, γ- and δ-H), 3.29 and 3.39 (2x d, *J*=6.2, 1H, OH, diast), 3.69 (s, 3H, OCH_3), 3.87-4.07 (m, 3H, β-H and ε-H), 4.17 and 4.26 (2x m, 1H, α-H, diast), 4.90 and 5.07 (2x d, *J*=9.8, 1H, δ-NH, diast), 5.17 (s, 2H, O$\underline{CH_2}$Ph), 5.26 (s, 2H, O$\underline{CH_2}$Ph), 7.25-7.42 (m, 10H, aryl), 9.28 and 9.46 (2x bs, 2H, ω- and ω'-NH).

2(*R,S*)-Hydroxy-3(*S*)-[(allyloxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)amino]-methyl]amino]hexanoic acid hydrochloride (61)

To a stirred solution of ester **56** (1.14 g, 2.00 mmol) in THF/MeOH/H_2O (60 mL, 4:1:1) was added LiOH·H_2O (0.25 g, 6.0 mmol). After 72 min, the pH was adjusted to ~9.5 (with 0.5 N HCl_{aq}). The basic solution was diluted with H_2O (total volume *ca* 350 mL), and extracted with Et_2O (3x 100 mL) to remove benzyl alcohol. The aqueous layer was acidified to pH≈ 3 (with 0.5 N HCl_{aq}), saturated with NaCl and extracted with EtOAc (3x 100 mL). The combined organic solutions were dried

(Na_2SO_4), filtrated and concentrated *in vacuo* to give **61** as a white foam (diastereomers (5:1), 0.92 g; 100%).

^{1}H-NMR (DMSO-d_6): 1.35-1.68 (m, 4H, γ- and δ-H), 3.10-3.42 (m, 3H, ε-H, OH), 3.80 (m, 1H, β-H), 3.98 (m, 1H, α-H), 4.48 (m, 2H, $OC\underline{H}_2CH{=}CH_2$), 5.11-6.31 (m, 3H, $OCH_2CH{=}C\underline{H}_2$, $OC\underline{H}_2Ph$), 5.89 (m, 1H, $OCH_2C\underline{H}{=}CH_2$), 6.80 and 7.14 (2x d, *J*=9.0, 1H, β-NH, diast), 7.24-7.44 (m, 5H, aryl) and 8.42-9.00 (m, < 4H, guanidinium NH).

2(*R,S*)-Hydroxy-3(*S*)-[(*tert*-butyloxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)-amino]methyl]amino]hexanoic acid hydrochloride (62)

Prepared from ester **60** (3.56 g, 6.07 mmol) according to the same procedure as described for **61**, yielding **62** as a white foam (diastereomers (11:1), 2.84 g, 98.6%).

^{1}H-NMR (DMSO-d_6): 1.38 and 1.39 (s, 9H, $C(CH_3)_3$), 1.41-1.68 (m, 4H, γ- and δ-H), 3.20-3.50 (m, 3H, ε-H, OH), 3.78 (m, 1H, β-H), 3.98 (m, 1H, α-H), 5.22 (s, 2H, $OC\underline{H}_2Ph$), 6.30 and 6.67 (2x bd, 1H, *J*=8.7 β-NH, diast), 7.32-7.48 (m, 10H, aryl), 8.42-9.00 (m, < 4H, guanidine NH) and 12.01 (bs, < 1H, COOH).

Methyl 2(*S*)-[[[1-(allyloxycarbonyl)pyrrolidin-2(*S*)-yl]carbonyl]amino]-5-[[imino[(phenyl-methoxycarbonyl)amino]methyl](phenylmethoxycarbonyl)amino]pentanoate (63)

To a stirred solution of *N*-Aloc proline (2.19 g, 11.0 mmol) in CH_2Cl_2 (25 mL) at 0 °C was added HOBt (2.30 g, 16.9 mmol) and DCC (2.50 g, 12.1 mmol). After 45 min, a solution of amine **52** (8.37 g, 11.0 mmol + 18.6 mmol TFA) and DiPEA (3.29 mL, 18.9 mmol) in CH_2Cl_2 (25 mL) was added. After another 60 min, the ice-bath was removed and the stirring was continued for 18 h. Subsequently, the solvent was removed under reduced pressure, the residue was taken up in EtOAc and filtrated. The organic solution was sequentially washed with, aqueous $NaHCO_3$ (5%, 3x), H_2O (2x), aqueous $KHSO_4$ (6%, 3x), H_2O (2x) and brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo* to give **63**, after purification by column chromatography (EtOAc/PE, 3:2), as an amorphous white powder (5.70 g; 81.3%).

^{1}H-NMR ($CDCl_3$): 1.35-1.68 (m, 8H, Arg β- and γ-H, Pro β- and γ-H), 3.42 (m, 2H, Pro δ-H), 3.61 (s, 3H, OCH_3), 4.00 (m, 2H, Arg δ-H), 4.25 (m, 1H, α-H), 4.49-4.69 (m, 3H, Pro α-H, $OC\underline{H}_2CH{=}CH_2$), 5.11 (s, 2H, $OC\underline{H}_2Ph$), 5.23 (s, 2H, $OC\underline{H}_2Ph$), 5.02-5.46 (overlapping m, 1H, $OCH_2CH{=}C\underline{H}_2$), 5.86 (m, 1H, $OCH_2C\underline{H}{=}CH_2$), 7.24-7.45 (m, 10H, aryl, Arg α-NH), 9.28 and 9.46 (2x bs, 2H, Arg ω- and ω'-NH).

2(*S*)-[[[1-(Allyloxycarbonyl)pyrrolidin-2(*S*)-yl]carbonyl]amino]-5-[[imino[(phenylmethoxy-carbonyl)amino]methyl]amino]pentanoic acid tetracitrate (64)

A solution of ester **63** (0.32 g, 0.50 mmol) and $LiOH{\cdot}H_2O$ (63.1 mg, 1.50 mmol) in THF/MeOH/H_2O (16.6 mL, 3:1:1) was stirred for 72 min. The reaction mixture was acidified to pH≈ 3 (with aqueous 1 M citric acid) and extracted with EtOAc (3x 25 mL). The combined organic solution were dried (Na_2SO_4), filtrated, and concentrated under reduced pressure to give **64** (0.7 g, 100%) as tetracitrate contaminated with 1 equiv of benzyl alcohol.

^{1}H-NMR (DMSO-d_6): 1.42-2.10 (m, 7H, Arg β- and γ-H, Pro β- and γ-H), 2.17 and 2.28 (AB-system, *J*=15.3, 16H, CH_2 citric acid), 3.18-3.50 (m, 2H, Arg δ-H, Pro δ-H), 4.10-4.31 (m, 2H, Arg α-H, Pro α-H), 4.46 (m, 2H, $OC\underline{H}_2CH{=}CH_2$), 5.03-5.31 (m, 4H, $OC\underline{H}_2Ph$, $OCH_2CH{=}C\underline{H}_2$), 5.82 (m, 1H, $OCH_2C\underline{H}{=}CH_2$), 7.25-7.46 (m, 5H, aryl), 8.28 (m, 1H, Arg α-NH) and 8.35-8.95 (m, < 4H, guanidinium NH).

Methyl 2(*R*)-[[2(*R,S*)-hydroxy-3(*S*)-[(*tert*-butyloxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)amino]methyl]amino]hexanoyl]amino]-3-phenylpropanoate (66)

To a stirred solution of acid **62** (0.30 g, 0.63 mmol) and HOBt (170 mg, 1.26 mmol) in THF (15 mL), at 0 °C, was added DCC (143 mg, 0.691 mmol). After 30 min, a solution of D-phenylalanine methyl ester hydrochloride (136 mg, 0.631 mmol) and TEA (89 μL, 0.64 mmol) in CH_2Cl_2 (5 mL) was added. After 1 h the cooling-bath was removed, and the reaction mixture was stirred for 18 h at room temperature. The reaction mixture was filtrated, concentrated *in vacuo*, and the residue was dissolved in EtOAc (40 mL). The resulting solution was sequentially washed with aqueous $NaHCO_3$ (5%, 3x), H_2O, aqueous $KHSO_4$ (6%, 3x), H_2O, aqueous $NaHCO_3$ (5%, 2x) and brine, dried (Na_2SO_4), filtrated, and concentrated *in vacuo* to give **66**, after purification by centrifugal chromatography ($CHCl_3$/MeOH, 96:4), as a white foam (diastereomers (6:1), 266 mg; 70.3%).

^{1}H-NMR ($CDCl_3$): 1.35 (s, 9H, Boc), 1.22-1.68 (m, 4H, Arg γ- and δ-H), 2.42-3.43 (m, 4H, hArg ε-H, Phe β-H), 3.75 and 3.77 (2x s, 3H, OCH_3, diast), 4.94 (m, 1H, hArg β-H), 4.04-4.17 (m, 1H, hArg α-H), 4.87 (m, 1H, Phe α-H), 4.97-5.15 (s, 2H, $OC\underline{H}_2Ph$), 5.42 and 5.49 (2x d, *J*=8.7, 1H, hArg β-NH), 6.67-7.87 (m, 13H, aryl, Phe α-NH, guanidine NH) and 8.02 (bs, 1H, Arg ε-NH).

Methyl 2(*R*)-[[2-oxo-3(*S*)-[(*tert*-butyloxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)amino]methyl]amino]hexanoyl]amino]-3-phenylpropanoate (67)

To a stirred solution of **66** (90 mg, 0.15 mmol) and HOAc (10 μL, 0.17 mmol) in CH_2Cl_2 (1 mL) was added a solution of Dess-Martin periodinane (0.22 g, 0.52 mmol) in CH_2Cl_2 (2 mL). After 90 min, Et_2O (60 mL) and a solution of $NaHCO_3$ (0.25 g) and $Na_2SO_3 \cdot 5H_2O$ (0.75 g) in H_2O (10 mL) were added. The mixture was shaken vigorously to give a clear bi-phasic system. The layers were separated, the aqueous layer was extracted with Et_2O (40 mL), and the combined etheral solutions were washed with aqueous $NaHCO_3$ (5%) and brine, dried (Na_2SO_4), filtrated, and concentrated *in vacuo* to give **67** as a white foam (65 mg; 72.5%).

^{1}H-NMR ($CDCl_3$): 1.40 and 1.42 (2x s, 9H, Boc), 1.29-1.87 (m, 4H, kArg γ- and δ-H), 2.95-3.27 (m, 4H, kArg ε-H, Phe β-H), 3.73 (m, 3H, OCH_3), 4.85 (m, 1H, Phe α-H), 4.93-5.15 (m, 3H, kArg γ- and β-H, $OC\underline{H}_2Ph$), 5.46 (m, 1H, kArg β-NH) and 6.96-7.45 (m, 13H, aryl, Phe α-NH, guanidine NH).

Methyl 2(*R*)-[[2(*R,S*)-hydroxy-3(*S*)-[(*tert*-butyloxycarbonyl)amino]-6-[[imino(amino)methyl]amino]hexanoyl]amino]-3-phenylpropanoate hydrochloride (68)

A solution of **66** (100 mg, 0.167 mmol) and aqueous HCl (1.09 M, 154 μL, 0.169 mmol) in MeOH (3.5 mL) was stirred vigorously with Pd/C (5%, 20 mg) in a H_2 atmosphere. After the H_2 uptake had ceased the reaction mixture was filtrated, and the solvent was removed under reduced pressure, yielding **68** as a white foam (diastereomers (6:1), 80 mg, 95.6%).

^{1}H-NMR ($CDCl_3$): 1.34 and 1.38 (2x s, 9H, Boc, diast), 1.36-1.82 (m, 4H, Arg γ- and δ-H), 3.02-3.17 (m, 4H, hArg ε-H, Phe β-H), 3.60 and 3.61 (2x s, 3H, OCH_3, diast), 3.69 (m, 1H, hArg β-H), 3.89 and 4.12 (2x m, 1H, hArg α-H, diast), 4.59 (m, 1H, Phe α-H), 5.87 and 5.91 (2x d, *J*=5.1, 1H, OH), 6.10 (d, *J*=8.8, 1H, hArg β-NH), 6.80-7.48 (m, 9H, aryl, guanidinium NH), 7.60 (m, 1H, Arg ε-NH) and 7.79 (d, *J*=5.1, 1H, Phe α-NH).

Methyl 2(*R,S*)-hydroxy-3(*S*)-[[2-(trimethylsilyl)ethoxycarbonyl]amino]-6-[[imino[(phenylmethoxycarbonyl)amino]methyl](phenylmethoxycarbonyl)amino]hexanoate (69)

To a stirred solution of amino ester **58** (8.62 g, 17.7 mmol) in MeCN (90 mL) was added Teoc-OPnp[25a] (5.03 g, 17.6 mmol) and DMAP (30 mg, 0.25 mmol). The reaction mixture was stirred

for 28 h at room temperature, diluted with EtOAc (300 mL), and sequentially washed with aqueous Na_2CO_3 (1 M, 6x), H_2O (2x) and brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo* to furnish a yellow oil. The crude product was purified by column chromatography (CH_2Cl_2/EtOAc, 95:5) to give **69** as a thick yellowish oil (diastereomers (11:2), 4.83 g; 43.3%).

^{1}H-NMR ($CDCl_3$): –0.01 (s, 9H, $Si(CH_3)_3$), 0.91 (m, 2H, CH_2Si), 1.45-1.80 (m, 4H, γ- and δ-H), 3.30 and 3.32 (2x m, 1H, OH, diast), 3.68 (s, 3H, OCH_3), 3.88-4.16 (m, 5H, β-H, ε-H and $C\underline{H}_2CH_2Si$), 4.19 and 4.28 (2x dd, *J*=4.7 and 3.4 (1.4), 1H, α-H, diast), 5.08 and 5.27 (2x d, *J*=8.4, 1H, β-NH, diast), 5.11 (s, 2H, $OC\underline{H}_2Ph$), 5.21 (s, 2H, $OC\underline{H}_2Ph$), 7.24-7.45 (m, 10H, aryl), 9.26 and 9.41 (bs, 2H, ω- and ω'-NH). FAB-HRMS: calcd for $[C_{30}H_{42}N_4O_6Si + H]^+$ 631.2799, found 631.2743.

2(*R,S*)-Hydroxy-3(*S*)-[[2-(trimethylsilyl)ethoxycarbonyl]amino]-6-[[imino[(phenylmethoxy-carbonyl)amino]methyl]amino]hexanoic acid hydrochloride (70)

To a stirred solution of **69** (1.98 g, 3.17 mmol) in THF/MeOH/H_2O (99 mL, 4:1:1) was added $LiOH{\cdot}H_2O$ (0.45 g, 9.6 mmol). After 72 min at room temperature, the pH of the reaction mixture was adjusted to pH≈ 8 (with 1N HCl_{aq}). Subsequently H_2O (500 mL) was added and the clear solution was extracted with Et_2O (2x 250 mL) to remove benzyl alcohol. The aqueous layer was acidified to pH≈ 2 (with 1N HCl_{aq}), saturated with NaCl, and extracted with EtOAc (3x 250 mL). The combined EtOAc solutions were dried (Na_2SO_4), filtrated and concentrated *in vacuo* to give **70** as a white foam (diastereomers (6:1), 1.40 g; 85.1%).

^{1}H-NMR (DMSO-d_6): –0.01 (s, 9H, $Si(CH_3)_3$), 0.92 (m, 2H, CH_2Si), 1.33-1.66 (m, 4H, γ- and δ-H), 3.29 (m, 2H, ε-H), 3.81 (m, 1H, β-H) , 3.92-4.12 (m, 3H, α-H and $C\underline{H}_2CH_2Si$), 5.22 (s, 2H, $OC\underline{H}_2Ph$), 5.56 and 5.92 (2x d, *J*=9.4, 1H, β-NH, diast), 7.33-7.48 (m, 5H, aryl), 8.33-9.94 (m, 3H, ε-NH_2^+, ω-NH and ω'-NH) and 12.05 (bs, 1H, CO_2H). ^{13}C-NMR (DMSO-d_6): –1.4 ($Si(CH_3)_3$), 17.3 (CH_2Si), 24.9 (δ), 28.5 (γ), 40.2 (ε), 53.0 (β), 61.7 ($O\underline{C}H_2CH_2Si$), 67.7 ($O\underline{C}H_2Ph$), 71.8 and 72.3 (α, diast), 128.0 (aryl-4), 128.3 (aryl-3,5), 128.6 (aryl-2,6), 135.2 (aryl-1), 152.8 (C(O)O, Z), 153.3 (C=N), 156.1 (C(O)O, Teoc) and 174.0 (CO_2H). FAB-HRMS: calcd for $[C_{21}H_{34}N_4O_7Si + H]^+$ 483.2275, found 483.2289.

2(*R,S*)-Hydroxy-3(*S*)-[[2-(trimethylsilyl)ethoxycarbonyl]amino]-6-[[imino(amino)methyl]-amino]hexanoic acid hydrochloride (71)

A solution of **70** (1.30 g, 2.50 mmol) in MeOH (12 mL) was stirred vigorously with Pd/C (5%, 130 mg) in a H_2 atmosphere until gas uptake ceased. Filtration and evaporation of the solvent *in vacuo* yielded **71** as a white foam (diastereomers (6:1), 0.85 g; 88.4%).

^{1}H-NMR (DMSO-d_6): –0.01 (s, 9H, $Si(CH_3)_3$), 0.91 (m, 2H, CH_2Si), 1.28-1.37 (m, 4H, γ- and δ-H), 3.07 (m, 2H, ε-H), 3.79 (m, 1H, β-H) , 3.90-4.18 (m, 3H, α-H and $C\underline{H}_2CH_2Si$), 6.58 and 6.90 (2x d, *J*=9.4, 1H, β-NH, diast) and 6.85-8.01 (m, 5H, ε-NH_2^+, ω-NH, ω'-NH and CO_2H). ^{13}C-NMR (DMSO-d_6): –1.3 ($Si(CH_3)_3$), 17.3 (CH_2Si), 25.5 (δ), 28.6 (γ), 40.2 (ε), 51.5 (β), 61.7 ($O\underline{C}H_2CH_2Si$), 71.8 and 72.0 (α, diast), 156.1 (C(O)O, Teoc), 157.0 (C=N) and 174.0 (CO_2H). FAB-HRMS: calcd for $[C_{13}H_{28}N_4O_5Si + H]^+$ 349.1907, found 349.1864.

Methyl 2(*R,S*)-hydroxy-3(*S*)-[[2-(trimethylsilyl)ethoxycarbonyl]amino]-6-[[imino[(1-adamantoxycarbonyl)amino]methyl](1-adamantoxycarbonyl)amino]hexanoate (72)

To a stirred solution of **71** (10.5 g, 27.3 mmol) in 1,4-dioxane (17 mL) at 0 °C was added a solution of NaOH (5.46 g, 0.132 mol) in H_2O (55 mL). An ice-cold solution of Adoc-Cl[28,29] (max 75 mmol) in 1,4-dioxane (25 mL), and $NaOH_{aq}$ (2 M, 82.5 mL) were added simultaneously over a period of 1 h.

Subsequently, ice-cold H_2O (75 mL) was added to the viscous reaction mixture, and stirring was continued for 3 h at 5 °C. The reaction mixture was partitioned between EtOAc (300 mL) and aqueous $KHSO_4$ (6%, 300 mL), the layers were separated, and the aqueous phase was extracted with EtOAc (2x 150 mL). The combined organic phases were sequentially washed with aqueous $KHSO_4$ (6%, 2x), H_2O and brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo* to give crude **73**, which was directly converted to methyl ester **72** with diazomethane as described for **46**. Column chromatography (CH_2Cl_2/EtOAc, 9:1) yielded **72** as a thick colourless oil (diastereomers (5:1), 8.38 g; 42.7%).
^{1}H-NMR ($CDCl_3$): –0.01 (s, 9H, $Si(CH_3)_3$), 0.93 (m, 2H, CH_2Si), 1.41-1.98 (m, 16H, γ-, δ-H and CH_2 Adoc), 2.03-2.24 (m, 18H, CH_2 Adoc and CH Adoc), 3.75 (s, 3H, OCH_3), 3.76 (m, 1H, OH), 3.88-4.18 (m, 5H, β-, ε-H and $C\underline{H}_2CH_2Si$), 4.28 and 4.38 (2x m, 1H, α-H, diast), 5.70 and 5.90 (2x d, *J*=9.0, 1H, β-NH, diast) and 9.08-9.44 (m, 2H, ω- and ω'-NH). ^{13}C-NMR ($CDCl_3$): –1.7 ($Si(CH_3)_3$), 17.5 (CH_2Si), 24.9 (δ), 27.4 (γ), 30.6 (CH, Adoc), 30.7 (CH, Adoc), 35.5 (CH_2, Adoc), 36.1 (CH_2, Adoc), 41.1 (2 x CH_2, Adoc), 44.1 (ε), 52.4 (OCH_3), 53.2 (β), 62.9 ($\underline{C}H_2CH_2Si$), 72.5 and 73.4 (α, diast), 78.9 (OC, Adoc), 83.7 (OC, Adoc) 154.3 (C(O)O, Adoc), 156.7 (C(O)O, Teoc), 160.5 (C(O)O, Adoc), 162.9 (C=N), 172.8 and 173.7 ($\underline{C}O_2CH_3$). FAB-HRMS: calcd for $[C_{36}H_{58}N_4O_9Si + H]^+$ 719.4051, found 719.3986.

2(*R,S*)-Hydroxy-3(*S*)-[[2-(trimethylsilyl)ethoxycarbonyl]amino]-6-[[imino[(1-adamantoxy-carbonyl)amino]methyl](1-adamantoxycarbonyl)amino]hexanoic acid (73)

To a stirred solution of **72** (103 mg, 0.14 mmol) in THF/MeOH/H_2O (4.4 mL, 4:1:1) was added $LiOH{\cdot}H_2O$ (18 mg, 0.42 mmol). After 75 min, at room temperature, the pH of the reaction mixture was adjusted to pH≈ 3 (with aqueous 6% $KHSO_4$). Subsequently, H_2O (35 mL) was added and the clear solution was extracted with CH_2Cl_2 (3x 20 mL). The combined organic phases were washed with brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo* to give **73** as a white foam (diastereomers (8:1), 91 mg; 90.1%).
^{1}H-NMR ($CDCl_3$): –0.01 (s, 9H, $Si(CH_3)_3$), 0.96 (m, 2H, CH_2Si), 1.48-1.82 (m, 16H, γ-, δ-H and CH_2 Adoc), 2.02-2.29 (m, 18H, CH_2 and CH Adoc), 3.70-4.12 (m, 5H, β-, ε-H and $C\underline{H}_2CH_2Si$), 4.25 and 4.31 (bs, 1H, α-H, diast), 5.60 and 5.79 (2x d, *J*=9.0, 1H, β-NH, diast). Between 7.00-10.6 ppm several very broad signals were present (guanidine-NH's, OH and CO_2H ?); integrals were inaccurate because of the broadness of the signals.

2(*S*)-[(Phenylmethoxycarbonyl)amino]-5-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxy-carbonyl)amino]methyl]amino]pentanoic acid (78)

To a stirred suspension of Cu($N^{\omega,\omega'}$-(bis-Boc)arginine)$_2$[32,33] (**77**) (73.4 g, 90.6 mmol) in H_2O (350 mL) was added EDTA·4Na·2H_2O (55.3 g, 0.109 mol) and $NaHCO_3$ (36.0 g, 0.426 mol). Subsequently, Z-Cl (35.0 mL, 0.238 mol) in acetone (250 mL) was added dropwise, upon which a dark blue solution was obtained. After 5 h at room temperature, the acetone was evaporated under reduced pressure and the residue was dissolved in H_2O (1 L). The aqueous solution was acidified to pH≈ 3.0 (with aqueous 6% $KHSO_4$), and extracted with EtOAc (5x 200 mL). The organic phase was washed with H_2O and brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo*. Purification of the crude product by column chromatography (gradient from PE/EtOAc, 2:1 to PE/EtOAc/HOAc, 2:2:0.1) gave **78** as a colourless oil (55.6 g; 60.3%).
^{1}H-NMR ($CDCl_3$): 1.50 (s, 9H, Boc), 1.53 (s, 9H, Boc), 1.58-2.04 (m, 4H, β- and γ-H), 3.39 (m, 2H, δ-H), 4.42 (m, 1H, α-H), 5.10 (bs, 1H, $OC\underline{H}_2Ph$), 5.78 (d, *J*=8.3, 1H, α-NH), 7.29-7.39 (m, 5H, aryl)

and 8.43 (m, 1H, δ-NH).

2(*S*)-[(Allyloxycarbonyl)amino]-5-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)-amino]methyl]amino]pentanoic acid (79)

Prepared from Cu($N^{\omega,\omega'}$-(bis-Boc)arginine)$_2$[32,33] (**77**) (3.91 g, 4.82 mmol) and Aloc-Cl (1.39 g, 11.57 mmol) following the same procedure as described for the synthesis of **78**. Purification of the crude product by column chromatography (gradient from PE/EtOAc, 2:1 to PE/EtOAc/HOAc, 2:2:0.1) gave **79** as a glass (2.45 g; 55.4%).

^{1}H-NMR ($CDCl_3$): 1.40 (s, 9H, Boc), 1.42 (s, 9H, Boc), 1.45-2.00 (m, 4H, β- and γ-H), 3.38 (m, 2H, δ-H), 4.47 (m, 1H, α-H), 4.54 (d, *J*=5.8, 2H, $OC\underline{H}_2CH{=}CH_2$), 5.23 (dd, 2H, $OCH_2CH{=}C\underline{H}_2$), 5.73 (d, *J*=8.2 Hz, 1H, α-NH), 5.90 (ddt, 1H, $OCH_2C\underline{H}{=}CH_2$) and 8.38 (m, 1H, δ-NH).

Methyl 2(*S*)-[(phenylmethoxycarbonyl)amino]-5-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyl-oxycarbonyl)amino]methyl]amino]pentanoate (80)

Prepared from acid **78** (7.51 g, 14.8 mmol) following the same procedure as described for the synthesis of **46**, to give **80** as a glass (6.99 g; 90.6%).

^{1}H-NMR ($CDCl_3$): 1.45 (s, 18H, 2x Boc), 1.54-1.96 (m, 4H, β- and γ-H), 3.40 (m, 2H, δ-H), 3.75 (s, 3H, OCH_3), 4.37 (m, 1H, α-H), 5.10 (bs, 1H, $OC\underline{H}_2Ph$), 5.51 (d, *J*=8.4, 1H, α-NH), 7.29-7.39 (m, 5H, aryl), 8.28 (bt, *J*=5.0, 1H, δ-NH) and 11.45 (bs, 1H, ω-NH). ^{13}C-NMR ($CDCl_3$): 25.6 (γ), 27.1 ($OC(\underline{C}H_3)_3$), 27.4 ($OC(\underline{C}H_3)_3$), 28.3 (β), 39.3 (δ), 51.4 (OCH_3), 52.9 (α), 65.8 ($O\underline{C}H_2Ph$), 78.5 ($O\underline{C}(CH_3)_3$), 82.3 ($O\underline{C}(CH_3)_3$), 127.1 (aryl-4), 127.4 (aryl-3,5), 127.6 (aryl-2,6), 135.6 (aryl-1), 152.3 (C(O)O, Boc), 154.7 (C(O)O, Z), 154.7 (C=N), 162.6 (C(O)O, Boc) and 171.9 ($\underline{C}(O)OCH_3$). FAB-HRMS: calcd for $[C_{25}H_{38}N_4O_8 + H]^+$ 523.2768, found 523.2786.

2(*S*)-[(Phenylmethoxycarbonyl)amino]-5-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxy-carbonyl)amino]methyl]amino]pentanal (81)

Prepared from methyl ester **80** (6.22 g, 11.9 mmol) according to the same procedure as described for the synthesis of **47**. The residue was in dissolved benzene, and again concentrated *in vacuo*, to give **81** as a white foam (5.68 g; 96.9%).

^{1}H-NMR ($CDCl_3$): 1.39 (s, 9H, Boc), 1.43 (s, 9H, Boc), 1.47-1.96 (m, 4H, β- and γ-H), 3.35 (m, 2H, δ-H), 4.25 (m, 1H, α-H), 5.05 (bs, 1H, $OC\underline{H}_2Ph$), 5.90 (d, *J*=8.6, 1H, α-NH), 7.26-7.36 (m, 5H, aryl), 8.35 (bt, *J*=5.1, 1H, δ-NH), 9.54 (s, 1H, C(O)H) and 11.40 (bs, 1H, ω-NH). ^{13}C-NMR ($CDCl_3$): 25.2 (γ), 25.6 (β), 27.8 ($OC(\underline{C}H_3)_3$), 28.0 ($OC(\underline{C}H_3)_3$), 39.7 (δ), 59.7 (α), 66.9 ($O\underline{C}H_2Ph$), 79.2 ($O\underline{C}(CH_3)_3$), 83.1 ($O\underline{C}(CH_3)_3$), 127.9 (aryl-4), 128.0 (aryl-3,5), 128.3 (aryl-2,6), 136.1 (aryl-1), 153.0 (C(O)O, Boc), 156.1 (C(O)O, Z), 156.1 (C=N), 163.1 (C(O)O, Boc) and 199.2 (C(O)H). FAB-HRMS: calcd for $[C_{24}H_{36}N_4O_7 + H]^+$ 493.2662, found 493.2671.

1,1,1-Trismethylthio-3(*S*)-[(phenylmethoxycarbonyl)amino]-6-[[(*tert*-butyloxycarbonyl)-imino[(*tert*-butyloxycarbonyl)amino]methyl]amino]hexan-2(*R,S*)-ol (82)

n-Butyllithium (1.6 M in hexanes, 30.6 mL, 49.0 mmol) was added, over a period of 10 min to a stirred solution of tris(methylthio)methane (6.9 mL, 52.0 mmol) in THF (132 mL) at –65 °C. After 20 min a precipitate had formed, and a precooled (–65 °C) solution of aldehyde **81** (5.68 g, 11.5 mmol) in THF (50 mL) was added in 30 min, upon which the precipitate dissolved. Stirring was continued for 5 h. Subsequently, the reaction mixture poured onto a stirred mixture of saturated aqueous NH_4Cl/CH_2Cl_2 (400 mL, 1:12). The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (3x 100 mL). The combined organic phases were washed with H_2O and brine, dried ($MgSO_4$), filtrated

and evaporated to dryness *in vacuo*. Purification of the crude product by column chromatography (gradient from CH_2Cl_2 to CH_2Cl_2/EtOAc, 9:1) afforded **82** as a thick oil (diastereomers (9:1) 4.00 g; 53.6%).

^{1}H-NMR ($CDCl_3$): 1.46 (s, 18H, 2x Boc), 1.52-1.78 (m, 4H, β- and γ-H), 2.17 and 2.22 (2x s, 9H, $C(SCH_3)_3$, diast), 3.41 (m, 2H, ε-H), 4.69 and 3.92 (2x bs, 1H, α-H, diast), 4.12 (m, 1H, β-H), 4.98-5.12 (m, 2H, $OC\underline{H}_2Ph$), 5.56 (d, *J*=8.6, 1H, β-NH), 7.22-7.40 (m, 5H, aryl), 8.35 (bt, *J*=4.9, 1H, ε-NH) and 11.49 (bs, 1H, ω-NH). ^{13}C-NMR ($CDCl_3$): 13.9 ($C(S\underline{C}H_3)_3$), 25.4 (γ), 28.1 ($OC(\underline{C}H_3)_3$), 28.2 ($OC(\underline{C}H_3)_3$), 33.1 (δ), 40.6 (ε), 50.4 (β), 65.9 ($O\underline{C}H_2Ph$), 74.0 ($\underline{C}(SCH_3)_3$), 76.7 (α), 79.2 ($O\underline{C}(CH_3)_3$), 83.0 ($O\underline{C}(CH_3)_3$), 127.9 (aryl-4), 128.1 (aryl-3,5), 128.3 (aryl-2,6), 136.8 (aryl-1), 153.2 (C(O)O, Boc), 156.0 (C(O)O, Z), 156.1 (C=N) and 163.5 (C(O)O, Boc). FAB-HRMS: calcd for $[C_{28}H_{46}N_4O_7S_3 + H]^+$ 647.2607, found 647.2660.

Methyl 2(*R,S*)-hydroxy-3(*S*)-[(phenylmethoxycarbonyl)amino]-6-[[(*tert*-butyloxycarbonyl)-imino[(*tert*-butyloxycarbonyl)amino]methyl]amino]hexanoate (83)

A solution of orthothioester **82** (4.00 g, 6.18 mmol) in MeOH (118 mL) and H_2O (8.5 mL) was vigorously stirred with $HgCl_2$ (5.66 g, 20.8 mmol) and HgO (1.69 g, 7.82 mmol) for 72 h at rt. The reaction mixture was filtrated over celite and the residue was washed with CH_2Cl_2 (300 mL), MeOH (50 mL) and H_2O (50 mL). The bi-phasic filtrate was separated, and the aqueous layer was extracted with CH_2Cl_2 (100 mL). The combined organic phases was sequentially washed with, saturated aqueous NH_4OAc (3x 150 mL) and saturated aqueous NH_4Cl (2x 150 mL), dried (Na_2SO_4), filtrated, and concentrated under reduced pressure, to give, after purification by MPLC (EtOAc/PE, 2:3), **83** as white foam (diastereomers (9:1), 2.70 g; 79.0%)

^{1}H-NMR ($CDCl_3$): 1.47 (s, 18H, 2x Boc), 1.51-1.71 (m, 4H, β- and γ-H), 3.22-3.57 (m, 2H, ε-H, OH), 3.70 and 3.76 (2x s, 3H, OCH_3), 4.10 (m, 1H, β-H), 4.18 and 4.30 (2x bs, 1H, α-H, diast), 4.98-5.11 (m, 2H, $OC\underline{H}_2Ph$), 5.49 and 5.57 (2x bd, *J*=9.6, 1H, β-NH), 7.20-7.38 (m, 5H, aryl), 8.31 (bt, *J*=5.3, 1H, ε-NH) and 11.44 (bs, 1H, ω-NH). ^{13}C-NMR ($CDCl_3$): 25.8 and 26.0 (γ, diast, 27.8 ($OC(\underline{C}H_3)_3$), 27.9 ($OC(\underline{C}H_3)_3$), 28.2 (δ), 40.2 (ε), 52.6 (OCH_3), 53.4 (β), 66.5 and 66.7 ($O\underline{C}H_2Ph$, diast), 72.0 and 72.6 (α, diast) 79.1 ($O\underline{C}(CH_3)_3$), 83.0 ($O\underline{C}(CH_3)_3$), 127.7 (aryl-4), 121.8 (aryl-3,5), 128.3 (aryl-2,6), 136.5 (aryl-1), 153.1 (C(O)O, Boc), 155.9 (C(O)O, Z), 156.1 (C=N), 163.2 (C(O)O, Boc) and 173.6 ($\underline{C}(O)OCH_3$). FAB-HRMS: calcd for $[C_{26}H_{40}N_4O_9 + H]^+$ 553.2874, found 553.2867.

Methyl 2(*S*)-[(allyloxycarbonyl)amino]-5-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxy-carbonyl)amino]methyl]amino]pentanoate (84)

Prepared from acid **79** (1.41 g, 3.08 mmol) following the same procedure as described for the synthesis of **46**, to give **84** as a clear, faintly coloured oil (1.31 g; 84.7%).

^{1}H-NMR ($CDCl_3$): 1.46 (s, 18H, 2x Boc), 1.53-1.97 (m, 4H, β- and γ-H), 3.40 (m, 2H, δ-H), 3.74 (s, 3H, OCH_3), 4.37 (m, 1H, α-H), 4.57 (d, *J*=5.6, 2H, $OC\underline{H}_2CH=CH_2$), 5.25 (m, 2H, $OCH_2CH=C\underline{H}_2$), 5.52 (d, *J*=8.4, 1H, α-NH), 5.90 (m, 1H, $OCH_2C\underline{H}=CH_2$), 8.30 (bt, *J*=5.0, 1H, δ-NH) and 11.47 (bs, 1H, ω-NH).

2(*S*)-[(Allyloxycarbonyl)amino]-5-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)-amino]methyl]amino]pentanal (85)

Prepared from ester **84** (4.70 g, 9.95 mmol) according to the same procedure as described for the synthesis of **81**, to give **85** as a clear oil (3.37 g; 76.6%).

^{1}H-NMR ($CDCl_3$): 1.51 (s, 18H, 2x Boc), 1.58-2.06 (m, 4H, β- and γ-H), 3.46 (m, 2H, δ-H), 4.35 (m,

1H, α-H), 4.60 (d, *J*=5.2, 2H, $OC\underline{H}_2CH=CH_2$), 5.27 (m, 2H, $OCH_2CH=C\underline{H}_2$), 5.81-6.05 (m, 2H, $OCH_2C\underline{H}=CH_2$ and α-NH), 8.38 (bt, *J*=5.0, 1H, δ-NH), 9.62 (s, 1H, C(O)H) and 11.49 (bs, 1H, ω-NH).

1,1,1-Trismethylthio-3(*S*)-[(allyloxycarbonyl)amino]-6-[[(*tert*-butyloxycarbonyl)imino-[(*tert*-butyloxycarbonyl)amino]methyl]amino]hexan-(*R,S*)-ol (86)

Prepared from aldehyde **85** (3.37 g, 7.62 mmol) according to the same procedure as described for the synthesis of **82**, to give **86** as a white foam (3.28 g; 72.2%).

^{1}H-NMR ($CDCl_3$): 1.49 (s, 18H, 2x Boc), 1.53-1.77 (m, 4H, γ- and δ-H), 2.22 (s, 9H, $C(SC\underline{H}_3)_3$), 3.70 (bs, 1H, α-H), 4.15 (m, 1H, β-H), 4.56 (m, 2H, $OC\underline{H}_2CH=CH_2$), 5.23 (m, 2H, $OCH_2CH=C\underline{H}_2$), 5.55 (d, *J*=5.2 , 1H, β-NH), 5.92 (m, 2H, $OCH_2C\underline{H}=CH_2$), 8.34 (m, 1H, ε-NH) and 11.49 (bs, 1H, ω-NH).

Methyl 2(*R,S*)-hydroxy-3(*S*)-[(allyloxycarbonyl)amino]-6-[[(*tert*-butyloxycarbonyl)imino-[(*tert*-butyloxycarbonyl)amino]methyl]amino]hexanoate (87)

Prepared from orthothioester **86** (2.66 g, 4.46 mmol) according to the same procedure as described for the synthesis of **83**. The crude hydroxy ester was purified by column chromatography (CH_2Cl_2/ EtOAc, 7:3), to give **87** as a slightly yellowish oil which solidified upon standing (diastereomers (10:1), 0.34 g; 15.2%). The main product **88**, *i.e.* the hydroxy ester in which the Aloc group was oxymercurated was isolated as a white foam (diastereomers (1:1), 2.00 g; 61.1%).

87: ^{1}H-NMR ($CDCl_3$): 1.46 (s, 18H, 2x Boc), 1.50-1.76 (m, 4H, β- and γ-H), 3.23-3.60 (m, 2H, ε-H, OH), 3.73 and 3.79 (2x s, 3H, OCH_3, diast), 4.05 (m, 1H, β-H), 4.14 and 4.28 (2x bs, 1H, α-H, diast), 4.50 (m, 2H, $OC\underline{H}_2CH=CH_2$), 5.12 (m, 2H, $OCH_2CH=C\underline{H}_2$), 5.50 and 5.57 (2x d, *J*=9.6, 1H, β-NH, diast), 5.88 (m, 1H, $OCH_2C\underline{H}=CH_2$), 8.32 (bt, *J*=5.6, 1H, ε-NH) and 11.42 (bs, 1H, ω-NH). ^{13}C-NMR ($CDCl_3$): 25.6 and 25.9 (γ, diast), 27.7 ($OC(\underline{C}H_3)_3$), 28.0 ($OC(\underline{C}H_3)_3$), 28.5 (δ), 40.1 (ε), 52.4 (OCH_3), 53.2 (β), 65.2 and 65.4($O\underline{C}H_2CH=CH_2$, diast), 72.0 and 72.7 (α, diast), 78.4 ($O\underline{C}(CH_3)_3$), 82.8 ($O\underline{C}(CH_3)_3$), 117.1 and 117.4 ($OCH_2CH=\underline{C}H_2$, diast), 132.5 ($OCH_2\underline{C}H=CH_2$), 152.9 (C(O)O, Boc), 155.7 (C(O)O, Aloc), 156.0 (C=N), 163.1 (C(O)O, Boc), 172.7 and 173.5 ($\underline{C}(O)OCH_3$). FAB-HRMS: calcd for $[C_{22}H_{38}N_4O_9 + H]^+$ 503.2717, found 503.2698.

88: ^{1}H-NMR ($CDCl_3$): 1.48 (s, 18H, 2x Boc), 1.54-1.70 (m, 4H, β- and γ-H), 1.83-2.25 (m, 2H, $OCH_2CH(OCH_3)C\underline{H}_2HgCl$), 3.40 and 3.41 (2x s, 3H, $OCH_2CH(OC\underline{H}_3)CH_2HgCl$, diast), 3.46 (m, 2H, ε-H), 3.78 and 3.82 (2x s, 3H, OCH_3, diast), 3.82-4.42 (m, 5H, α-, β-H, $OC\underline{H}_2C\underline{H}(OCH_3)CH_2HgCl$), 5.30 and 5.43 (2x d, *J*=9.5, 1H, β-NH, diast), 8.37 (bt, *J*=5.5, 1H, ε-NH) and 11.46 (bs, 1H, ω-NH).

^{13}C-NMR ($CDCl_3$): 25.8 and 26.0 (γ), 27.8 ($OC(\underline{C}H_3)_3$), 28.1 ($OC(\underline{C}H_3)_3$), 28.8 (δ), 31.5 and 32.1 (CH_2 *Aloc-Hg*, diast), 40.3 (ε), 52.6 ($C(O)O\underline{C}H_3$), 53.5 and 53.6 (β, diast), 56.4 and 56.5 (OCH_3 *Aloc-Hg*), 71.9 (α), 78.3 and 78.4 (CH *Aloc-Hg*, diast), 79.1 and 79.1 ($O\underline{C}(CH_3)_3$, diast), 82.9 and 82.9 ($O\underline{C}(CH_3)_3$, diast), 153.0 (C(O)O, Boc),155.3 and 155.3 (C(O)O, *Aloc-Hg*, diast), 156.0 and 156.0 (C=N, diast), 163.2 and 163.2 (C(O)O, Boc, diast), 173.7 and 173.8 ($\underline{C}(O)OCH_3$, diast).

2(*R,S*)-Hydroxy-3(*S*)-[(phenylmethoxycarbonyl)amino]-6-[[(*tert*-butyloxycarbonyl)imino-[(*tert*-butyloxycarbonyl)amino]methyl]amino]hexanoic acid (89)

To a vigorously stirred solution of ester **83** (5.33 g, 9.64 mmol) in THF/MeOH/H_2O (313 mL, 4:1:1) was added finely powdered $LiOH\cdot H_2O$ (1.12 g, 26.7 mmol). After exactly 12 min, aqueous $KHSO_4$ (6%, 73 mL) was added, and THF was evaporated under reduced pressure. The residual, turbid liquid was diluted with H_2O (75 mL), acidified to pH≈ 2 (with aqueous 6% $KHSO_4$) and extracted with EtOAc (3x 100 mL). The combined organic phases were washed with H_2O and brine, dried (Na_2SO_4),

filtrated, and concentrated *in vacuo*, to afford pure **89** as a white foam (diastereomers (14:1), 5.01 g; 96.4%). Upon longer reaction times also the formation of cyclic carbamate **90** was observed.
89: ^{1}H-NMR ($CDCl_3$): 1.43 (s, 9H, Boc), 1.48 (s, 9H, Boc), 1.45-1.70 (m, 4H, γ- and δ-H), 3.35 (m, 2H, ε-H), 4.09 (m, 1H, β-H), 4.12 and 4.29 (2x m, 1H, α-H, diast), 4.98-5.12 (m, 2H, O$\underline{C}H_2$Ph), 6.68 (d, *J*=8.6, 1H, β-NH), 5.70 (bs, < 1H, C(O)OH), 7.20-7.41 (m, 5H, aryl) and 8.52 (m, 1H, ε-NH).
90: ^{1}H-NMR ($CDCl_3$): 1.41 (s, 9H, Boc), 1.47 (s, 9H, Boc), 1.45-1.70 (m, 4H, γ- and δ-H), 3.38 (m, 2H, ε-H), 3.90 (m, 1H, β-H), 4.44 (d, *J*=3.8, 1H, α-H), 6.79 (bs, 1H, β-NH), 7.52 (bs, 1H, C(O)OH) and 8.62 (m, 1H, ε-NH).

Methyl 2-oxo-4(*S, trans*)-[[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]-methyl]amino]propyl]-5-oxazolidinecarboxylate (91a)

A sample containing **89** and **90** (1.55 g, 2.62 mmol) obtained from treatment of ester **83** with LiOH during 72 min, was reacted with diazomethane as described for **46**. Purification of the crude product, by centrifugal chromatography (gradient from CH_2Cl_2/EtOAc, 95:5 to CH_2Cl_2/EtOAc, 90:10), gave hydroxy ester **83** (diastereomers (83:17), 0.91 g; 62.8 %) and *trans* oxazolidine **91a** as a white foam (0.40 g; 28.3 %).
91a: ^{1}H-NMR ($CDCl_3$): 1.45 (s, 18H, 2x Boc), 1.51-1.86 (m, 4H, γ- and δ-H), 3.30 (m, 2H, ε-H), 4.73 (s, 3H, OCH_3), 3.93 (m, 1H, β-H), 4.51 (d, *J*=4.7, 1H, α-H), 6.68 (bs, 1H, β-NH) and 8.40 (bt, *J*=5.3, 1H, ε-NH). ^{13}C-NMR ($CDCl_3$): 24.6 (γ), 27.8 (OC($\underline{C}H_3)_3$), 27.9 (OC($\underline{C}H_3)_3$), 31.9 (δ), 38.8 (ε), 52.7 (OCH_3), 55.1 (β), 77.5 (α), 79.7 (O$\underline{C}(CH_3)_3$), 83.2 (O$\underline{C}(CH_3)_3$), 151.9 (C(O)O, Boc), 156.1 (C=N), 156.8 (C(O)O, cyclic), 162.9 (C(O)O, Boc) and 169.3 ($\underline{C}(O)CH_3$). FAB-HRMS: calcd for $[C_{19}H_{32}N_4O_8 + H]^+$ 445.2298, found 445.2308.

3.7 References and Notes

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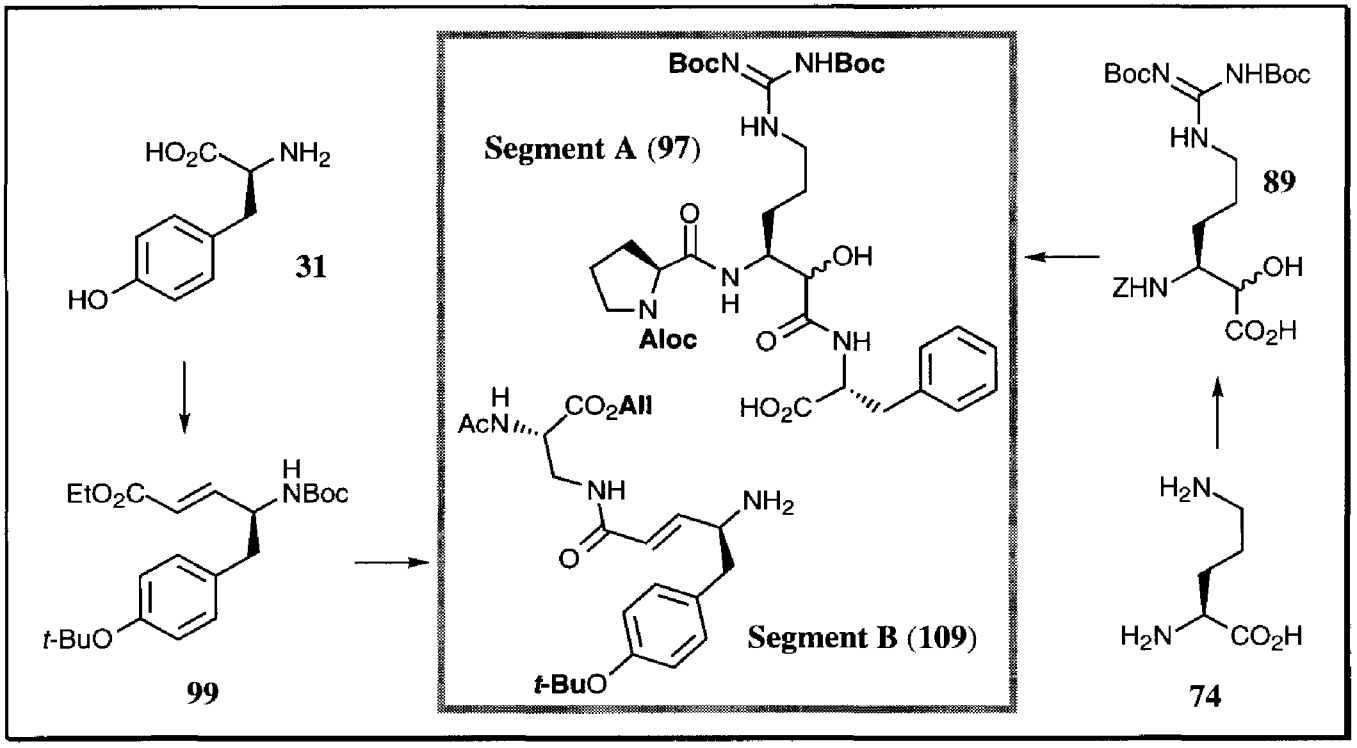
CHAPTER FOUR

Synthesis of the Key Intermediates: Segment A and Segment B



Abstract

In this chapter the synthesis of the key intermediates, Segment A and Segment B, is described. Of the two precursors for Segment A described in Chapter Three, β-homoarginine derivative **89** was successfully elaborated into tripeptide **97**. For the preparation of Segment B, a synthesis of α,β-unsaturated γ-amino ester **99** was devised starting from tyrosine (**31**). As it was found that protection of the hydroxyphenyl group (of vTyr) was necessary to prevent side-reactions during oxidation of the hydroxy amide unit in the penultimate step in our total synthesis, a procedure was developed to cleave the *N*-Boc group in the presence of a *t*-butyl ether to give **109**.

4.1. Introduction

As set out in Chapter 2.3, we planned to construct the macrocyclic ring of Cyclotheonamide by condensation of two fragments, a Pro-hArg-D-Phe tripeptide, *i.e.* Segment A, and a vTyr-Dpr dipeptide, *i.e.* Segment B. The condensation of the protected key fragments, by formation of the amide bond between the D-phenylalanine and the vinylogous tyrosine unit, would give a linear pentapeptide with a protected*C*- and *N*-terminus. Deprotection of these termini, consisting of a proline and a 2,3-diaminopropanoic acid unit, and subsequent cyclization of the linear pentapeptide then would furnish the protected macrocycle. To allow selective, simultaneous deprotection of *C*- and *N*-terminus, the protecting groups to be removed should of the same type, should be orthogonal to the other protecting groups present and should preferably be cleavable under neutral conditions.

The most conspicuous and synthetically most challenging part of the tripeptide is α-hydroxy-β-homoarginine unit, for which, as described in Chapter Three, two closely related derivatives were prepared, *viz.* N^{β}-Teoc, $N^{\varepsilon,\omega}$-(bis-Adoc)-α-hydroxy-β-homoarginine (**73**) and N^{β}-Z, $N^{\omega,\omega'}$-(bis-Boc)-α-hydroxy-β-homoarginine (**89**). In Section 4.2, the synthesis of protected Segment A is described starting from these β-homoarginine derivatives.

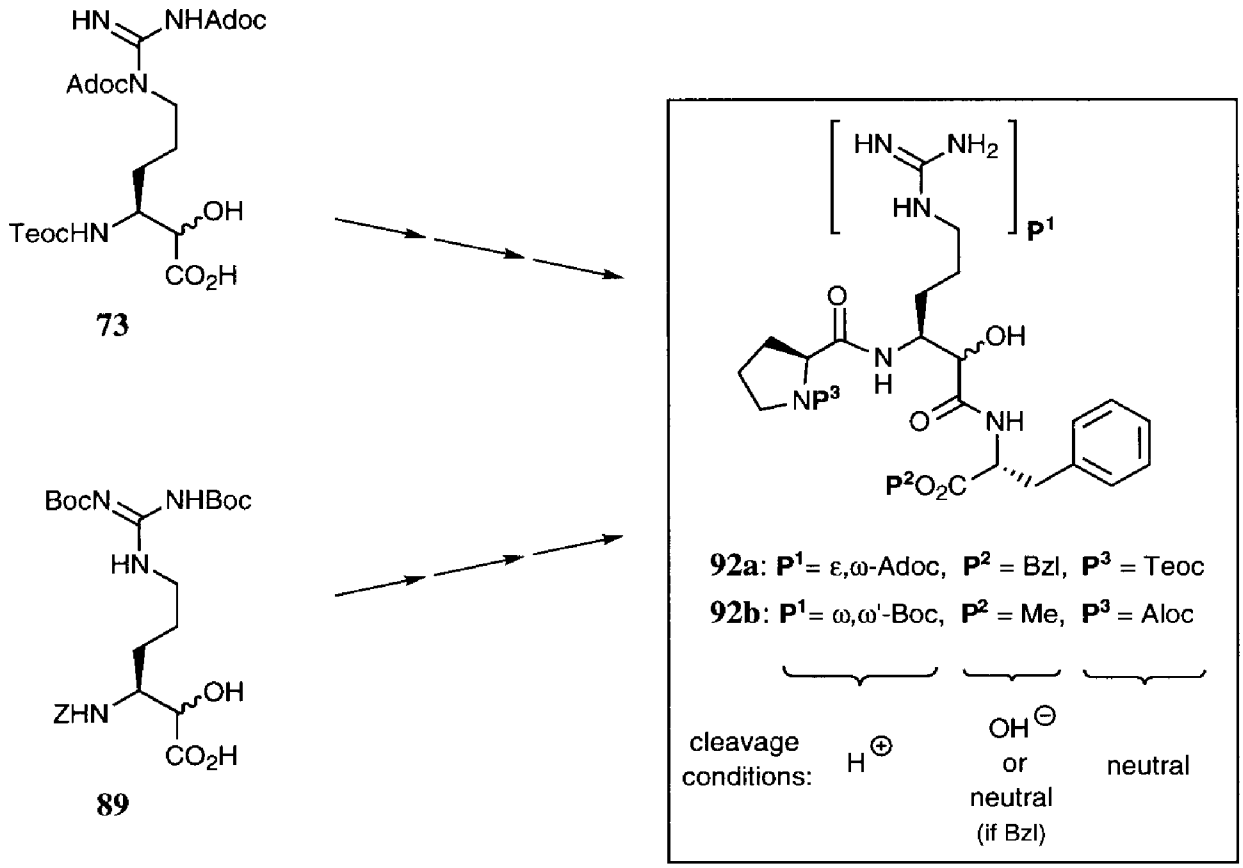
The second key intermediate, Segment B, features a vinylogous tyrosine and a N^{α}-acetyl-2,3-diaminopropanoic acid unit. In Section 4.3 the synthesis of protected Segment B from the properly protected amino acid constituents is described. Both non-proteinogenic amino acids were prepared from commercially available starting materials, *viz.* L-tyrosine (**31**) and L-aspartic acid (**101**).

4.2. Synthesis of Segment A

It was expected that both β-homoarginine derivatives **73** and **89** would be adequate building blocks for the synthesis of a protected Pro-hArg-D-Phe tripeptide **92a,b**, with different but, in principle, equivalent protecting groups [Scheme 4.1]. Common features of **73** and **89** are the presence of the two acid-labile protecting groups at the guanidino moiety, an unprotected hydroxyl group, and a free carboxylic acid group which is ready to be coupled to the D-phenylalanine unit. In both intermediates, the β-amino protecting groups were expected to be easily removable under neutral conditions to enable amide coupling with a suitable proline derivative.

The nature of the D-phenylalanine and proline protecting groups ($\mathbf{P^2}$ and $\mathbf{P^3}$, respectively) depicted in Scheme 4.1 will be discussed in the following sections. Cleavage of the carboxyl protecting group $\mathbf{P^2}$ would give, Segment A, suitable for further elaboration into Cyclotheonamide B

Scheme 4.1. α-Hydroxy-β-homoarginine derivatives **73** and **89** as building blocks for protected tripeptide **92a,b**.

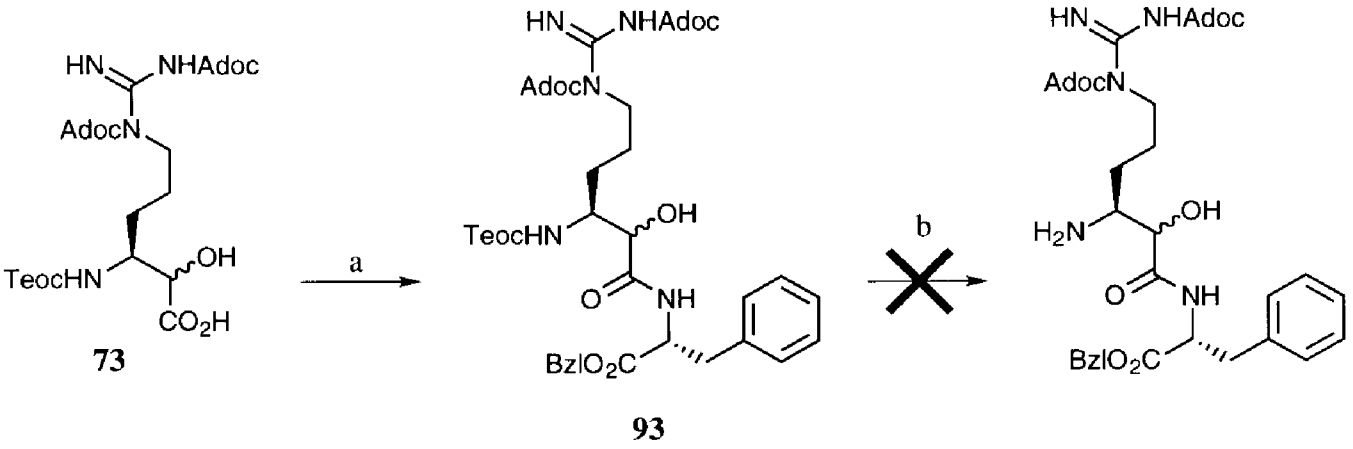


4.2.1. Approach to Segment A from N^{β}-Teoc, $N^{\varepsilon,\omega}$-(bis-Adoc)-α-hydroxy-β-homoarginine (73)

At the time we were studying the synthesis of Segment A starting from **73** we had still great difficulties in controlling the reactivity of the unprotected α-hydroxyl group of the β-homoarginine derivatives. As shown in Chapter 3.2.3, saponification of the arginine methyl ester in Pro-hArg dipeptide **59** [Scheme 3.7] resulted in cleavage of the amide bond, apparently by intramolecular nucleophilic attack of the hydroxyl group at that amide bond. Since the hydroxyl group could not be properly protected, we concluded that, in order to prevent side-reactions due to the reactivity of this hydroxyl group, basic reaction conditions *en route* to Cyclotheonamide had to be avoided. This implicated that the carboxylic acid group of the D-phenylalanine unit was to be protected with a group removable under neutral conditions, *e.g.* a benzyl group.

Thus, **73** was coupled with D-phenylalanine benzyl ester using DCC and HOBt to give dipeptide **93** [Scheme 4.2]. To enable coupling with proline, the Teoc group of dipeptide **93** had to be removed. Surprisingly, the benzyl ester was cleaved at a rate much faster than the Teoc group upon treatment with tetrabutylammonium fluoride (TBAF, 1 M in THF) at room temperature[1] or tetrabutylammonium chloride (TBAC)/potassium fluoride dihydrate in MeCN at 55 °C.[2] We ascribe this benzyl ester cleavage to an intramolecular attack of, again, the hydroxyl group.

Scheme 4.2. Attempted synthesis of tripeptide **92a** starting with α-hydroxy-β-homoarginine **73**.



a) DCC/HOBt, THF, 45 min, followed by D-phenylalanine benzyl ester·HCl/triethylamine (TEA), 63%; b) TBAF, 1 M in THF, rt; or TBAC/KF·$2H_2O$, MeCN, 55 °C.

Meanwhile, we had disclosed the route to **89**, and had solved the problem of the intramolecular reactivity of the hydroxyl group under basic conditions [Chapter 3.4.3]. Thus, the route to Segment A *via* β-homoarginine **73** was abandoned, and we focused our efforts on the synthesis of Segment A starting with **89**. However, in retrospect, elaboration of **73** into Segment A might have been feasible by using D-phenylalanine methyl ester (instead of the labile benzyl ester) in combination with the *fast hydrolysis* procedure, which was successfully used in the preparation of Segment A from **89**, as will be discussed in the next section.

4.2.2. Synthesis of Segment A (97) from N^{β}-Z, $N^{\omega,\omega'}$-(bis-Boc)-α-hydroxy-β-homoarginine (89)

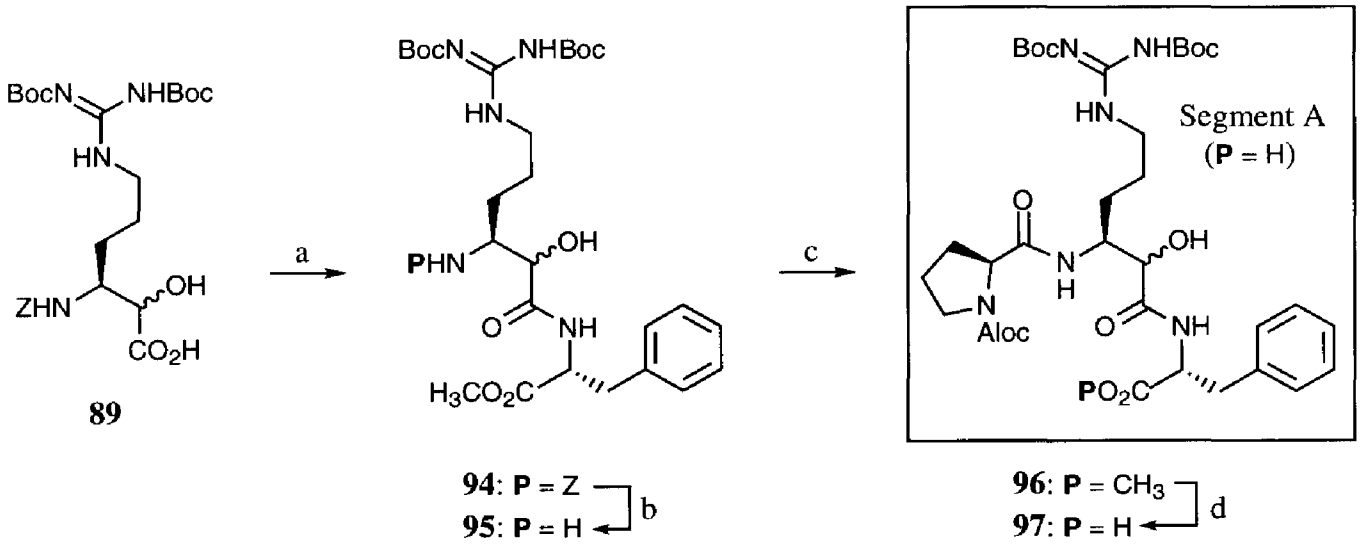
Fortunately, elaboration of **89** into Segment A was without further problems, once we had solved the problems originating from the reactivity of the hydroxyl group. Ester hydrolysis of peptides containing an α-hydroxy-β-homoarginine unit were now successfully conducted using the controlled, *fast hydrolysis* procedure described in Chapter 3.4.3.

Thus, β-homoarginine derivative **89** was coupled to D-phenylalanine methyl ester, using DCC and HOBt to furnish dipeptide **94** in 61% yield [Scheme 4.3]. Selective removal of the Z group by hydrogenolysis gave amine **95** in quantitative yield. Coupling of **95** with *N*-allyloxycarbonyl (Aloc) proline using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)[3,4] afforded tripeptide **96** in 79% yield.

The use of this advanced and very powerful coupling reagent simplifies peptide coupling procedures to a great extent. Mixing the amino acid derivatives with stoichiometric amounts of TBTU and DiPEA in CH_2Cl_2, gives the desired peptide after a reaction time of just 1.5-2 hours. The by-products, tetramethylureum and HOBt, are water soluble. In case of simple couplings, the pure peptides are obtained in essentially quantitative yields after aqueous work-up.[3]

Hydrolysis of the methyl ester of **96** with LiOH (12 min) gave tripeptide **97**, *i.e.* Segment A, in quantitative yield. Upon prolonged treatment of **96** with LiOH cleavage of the Pro-hArg bond was observed, presumably again through nucleophilic attack of the hydroxyl group [Chapter 3.2.3].

Scheme 4.3. Synthesis of Segment A (**97**).



a) DCC/HOBt, THF, 45 min, followed by D-phenylalanine methyl ester·HCl/DiPEA, 61%; b) H_2/Pd/C, MeOH, 100%; b) *N*-Aloc proline/TBTU/TEA, CH_2Cl_2, 2.5 h, 79%; d) LiOH, THF/MeOH/H_2O (3.6:1:1), rt, 12 min, 100%.

4.3. Synthesis of Segment B

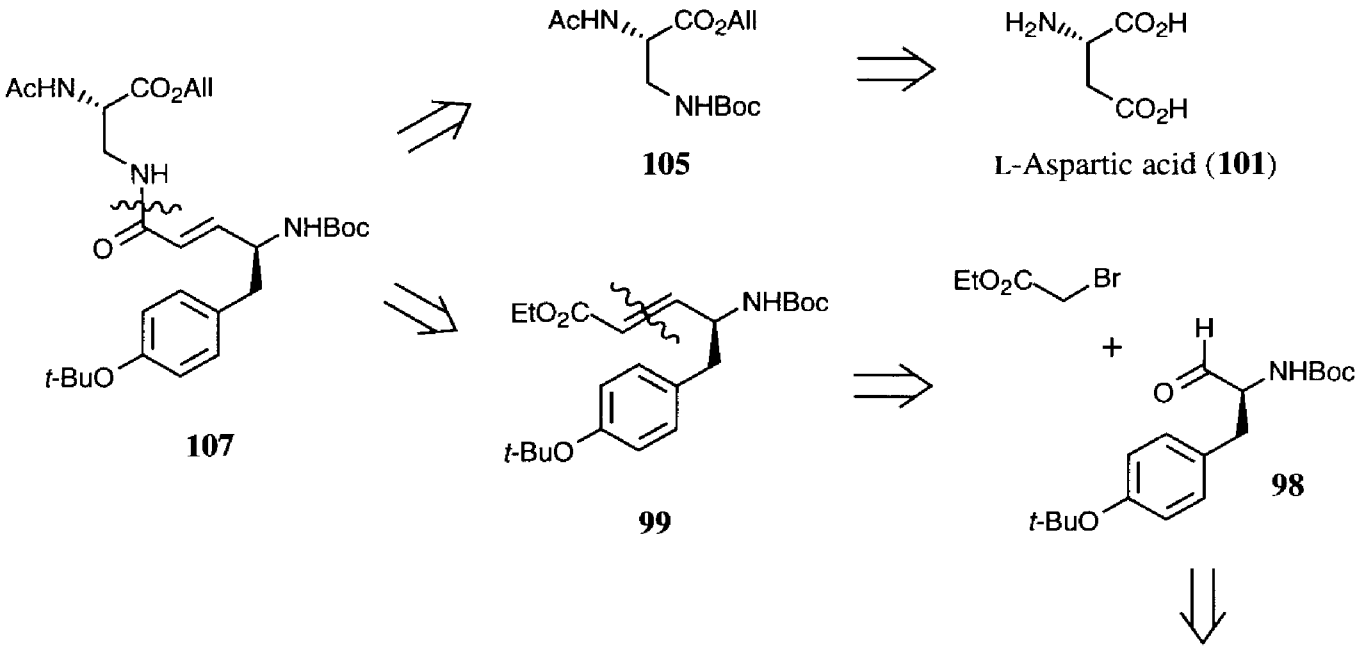
Now that we had worked out the nature of the protecting groups in Segment A (**97**), we could focus on selecting the protecting groups for Segment B, especially the one protecting the carboxylic acid group of the 2,3-diaminopropanoic acid unit [Scheme 4.4].

With an Aloc group on the nitrogen atom of the proline unit of **97**, it would be advantageous to protect the carboxylic acid group of the 2,3-diaminopropanoic acid unit of Segment B as an allyl ester to facilitate simultaneous Pd(0)-catalysed deprotection of the linear pentapeptide. Blocking of the α-amino group of 2,3-diaminopropanoic acid by an acetyl group (as present in the target molecule Cyclotheonamide B, see **105**) would allow regioselective coupling of the β-amino group with a vinylogous tyrosine derivative.

Originally, protection of the hydroxyphenyl moiety of the vinylogous tyrosine derivative was considered only important during preparation of the α,β-unsaturated γ-amino acid from tyrosine as we anticipated that we could find conditions for oxidation of the hydroxyl group of the β-homoarginine unit in the cyclopentapeptide that would tolerate the presence of an unprotected hydroxyphenyl group. As a result of this assumption, acid labile protecting groups, for both the amino and hydroxyphenyl group of the tyrosine unit, were selected.

In Scheme 4.4 a fully protected dipeptide **107**, based upon the above considerations, is depicted. Retrosynthetic analysis gives, after disconnection of the vTyr-Dpr peptide bond, two protected amino acid derivatives: vinylogous tyrosine **99**, to be prepared by a Wittig-type olefination from the protected tyrosine-derived aldehyde **98** [Section 4.3.1], and 2,3-diaminopropanoic acid derivative **105**, accessible *via* a Curtius rearrangement starting from aspartic acid (**101**) [Section 4.3.2].

Scheme 4.4. Retrosynthetic analysis of the protected vTyr-Dpr dipeptide **107**.



Treatment of dipeptide **107** with acid (*e.g.* TFA/H_2O or HCl/Et_2O), which cleaves both the Boc group and the *t*-butyl ether, should give the second key intermediate, Segment B. Unfortunately, it was found that the conditions for oxidation of the α-hydroxy-β-homoarginine moiety to the corresponding α-keto amide are incompatible with an unprotected hydroxyphenyl group. At this point we decided to study the possibility of selective *N*-Boc cleavage in the presence of an aryl *t*-butyl ether rather than devising a new synthesis for Segment B with proven orthogonal *O,N*-protection. It was gratifying to find that, this selective and, as far as we know, unprecedented *N*-Boc cleavage in the presence of the acid-labile aryl *t*-butyl ether was achieved by treatment of *e.g.* **107** with trimethylsilyl trifluoromethanesulfonate/ 2,6-lutidine [Section 4.3.3].

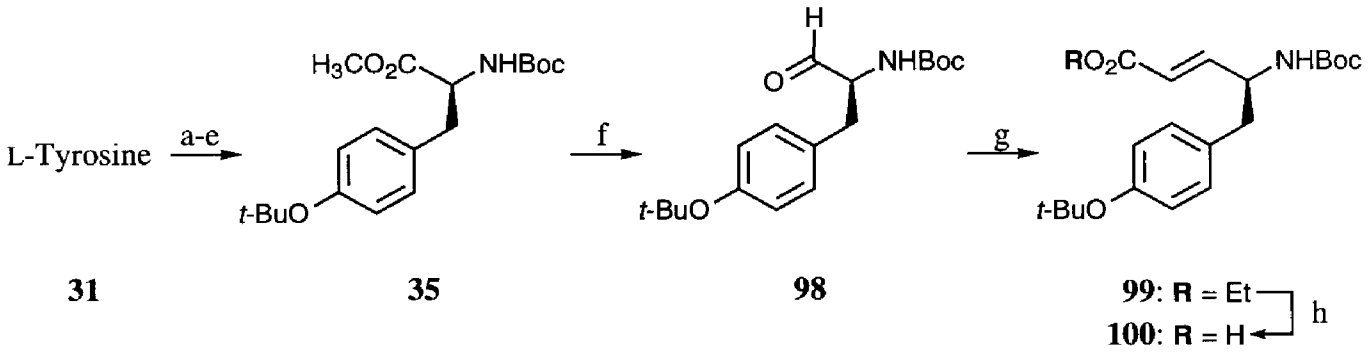
4.3.1. Synthesis of vinylogous tyrosine derivative 99

Preparation of the starting material **35** [Scheme 4.5] was achieved by a five-step sequence starting with tyrosine (**31**), employing literature procedures.[5]

Reduction of **35** with DiBAH (–72 °C, 20-25 min) gave the racemization-prone α-amino aldehyde **98**. The crude aldehyde was converted immediately by a Wadsworth-Emmons olefination using triethyl phosphonoacetate/NaH, at –50→10 °C, to give exclusively the *E*-alkene **99** in 94% yield (from **35**).[6,7] Noteworthy in this connection is a paper of Metternich *et al.* on the synthesis of an analogue of **99**, with a TBDMS ether instead of a *t*-butyl ether.[8] In their hands, reaction of the tyrosine derived aldehyde with triethyl phosphonoacetate/*n*-BuLi at –78 °C gave a mixture of *E*- and *Z*-alkenes whose ratio appeared to be dependent on the reaction temperature. Upon elevated temperatures (0 °C) the initially formed *E*- and *Z*-mixture isomerized *via* an intramolecular Michael-addition induced re-arrangement to give mainly the *E*-alkene. However, under the conditions we used for the preparation of **99**, solely *E*-alkene formation was observed.

Hydrolysis of the ethyl ester of **99**, with NaOH in dioxane/H_2O, gave acid **100** as a colourless crystalline solid, in essentially quantitative yield, ready to be coupled to a suitable diaminopropanoic acid derivative.

Scheme 4.5. Synthesis of vinylogous tyrosine derivative **100**.



a) $SOCl_2$, MeOH, rt, 2 h, reflux 0.5 h, 100%; b) Z-Cl/Na_2CO_3, CH_2Cl_2/H_2O, 0 °C→rt, 2.5 h, 98%; c) isobutene/H_2SO_4, CH_2Cl_2, 36 h, 89%; d) H_2/Pd/C, MeOH, THF, 6 h, 92%; e) Boc_2O, DMF, 60 °C, 0.5 h, 91%; f) DiBAH, CH_2Cl_2, –72 °C, 20 min; g) triethyl phosphonoacetate/NaH, THF, –50 → 10 °C, 2.5 h, 94% (two steps); h) NaOH, dioxane/H_2O, 14 h, 99%.

At first, the sequence depicted in Scheme 4.5 was started with D-tyrosine, as the vinylogous tyrosine unit of Cyclotheonamide was originally assigned the (*R*)-configuration by Fusetani *et al.*.[9] However, when Schreiber and Hagihara disclosed the first total synthesis of Cyclotheonamide B and concomitantly revised the stereochemistry of the vinylogous tyrosine unit as being *S*,[10] the synthesis of our vinylogous tyrosine derivative was repeated starting with L-tyrosine.

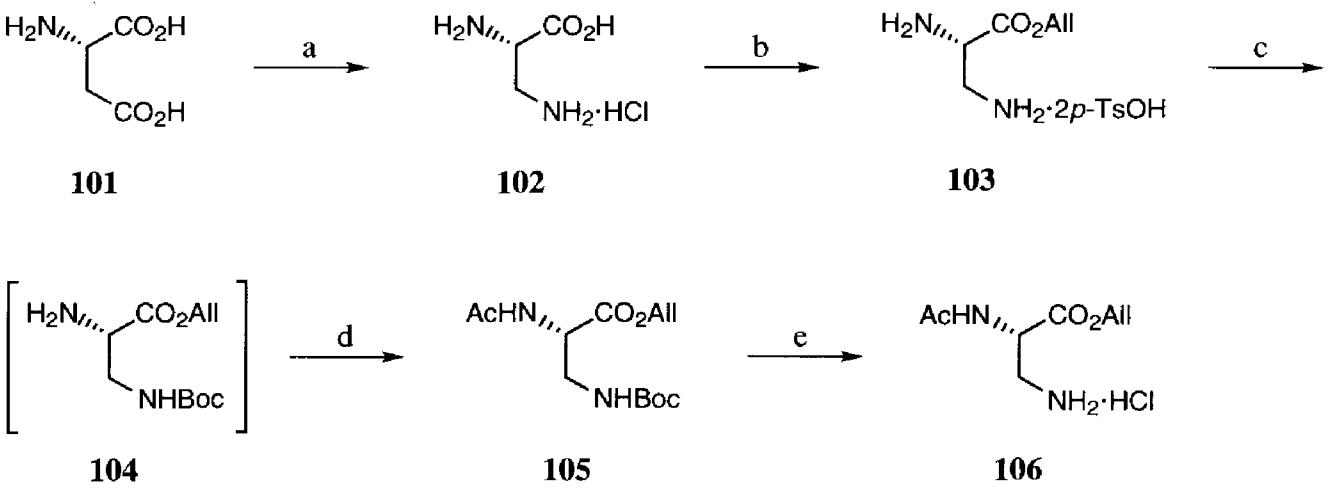
4.3.2. Synthesis of diaminopropanoic acid derivative 105

Although L-2,3-diaminopropanoic acid is not a proteinogenic amino acid, it is a constituent of several natural occurring antibiotics as well as other biologically active molecules.[11] Since L-2,3-diaminopropanoic acid is rather expensive (*ca* fl 300,– per gram), and its preparation by a Curtius rearrangement of the inexpensive L-aspartic acid (**101**) is rather straightforward,[12] we decided to start our synthesis of the diaminopropanoic acid derivative with L-aspartic acid (**101**) [Scheme 4.6].

In order to selectively introduce the acetyl group at the α-amino group of 2,3-diaminopropanoic acid it is necessary to protect the β-amino group first. In a recent paper on selective protection of diaminopropanoic acid, the introduction of a Boc group with a regioselectivity of N^{β} : N^{α} = 8:1 was reported;[13] treatment of methyl 2,3-diaminopropanoate with Boc_2O/TEA under high-dilution conditions gave, after purification by chromatography, the N^{β}-Boc derivative in 70% yield.
This method, after modification, was shown to work out also nicely starting with **103**, prepared from **102** by esterfication with allyl alcohol/*p*-TsOH in refluxing benzene. In our first attempt, aqueous work-up followed by chromatographic purification of the isomeric mixture (N^{β} : N^{α} = 8:1, crude yield 48%) gave the desired **104** in only 11% yield. Subsequent reaction of **104** with acetyl chloride/pyridine in MeCN furnished the fully protected diaminopropanoic acid derivative **105** in a rather disappointing overall yield of 6% (from ester **103**). In an effort to optimize this reaction sequence, the isomeric allyl

N-Boc diaminopropanoate mixture was not worked-up, but treated directly with acetyl chloride. Subsequent aqueous work-up and chromatography now furnished **105** in 64% yield.
To allow coupling with vinylogous tyrosine derivative **100**, the N^{β}-Boc group in **105** was removed by treatment with an etheral solution of HCl. Recrystallization from ethyl acetate afforded **106** as a colourless, crystalline solid in 86% yield.

Scheme 4.6. Synthesis of allyl N^{α}-acetyl-L-2,3-diaminopropanoate (**106**) from L-aspartic acid (**101**).



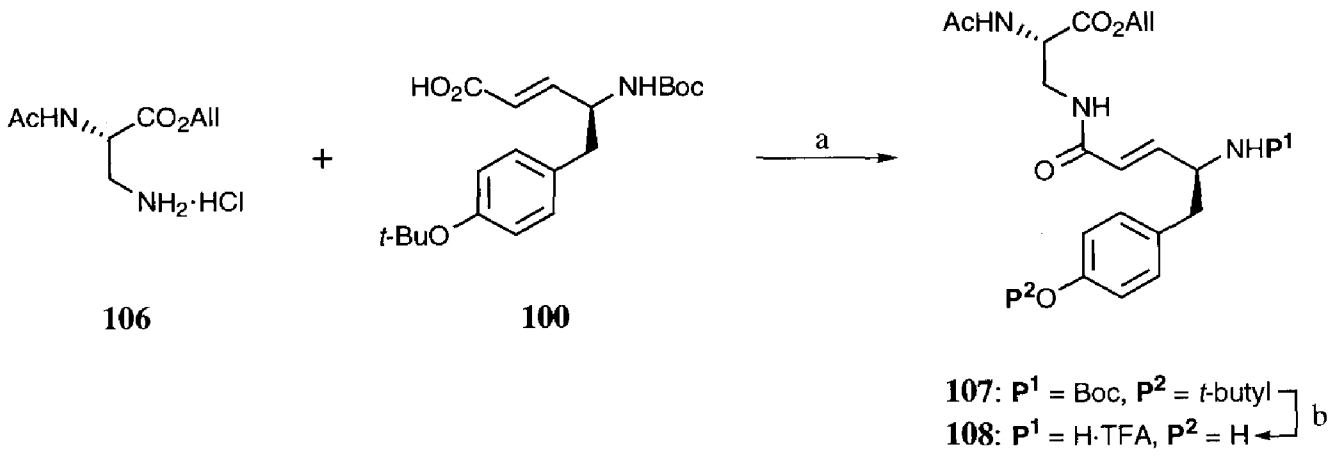
a) NaN_3, 30% oleum/$CHCl_3$, 58 °C, 4 h, followed by H_2O, 58%; b) allyl alcohol/*p*-TsOH, benzene, reflux, 5h, 77%; c) Boc_2O/TEA, CH_2Cl_2, –68→2 °C, 4 h, d) acetyl chloride, 2→20 °C, 16 h, 64% (two steps); e) 3M HCl in Et_2O, 0 °C→rt, 1 h, 86%.

4.3.3. Synthesis of 107 from 100 and 106, and selective deprotection to give Segment B

Coupling of vinylogous tyrosine **100** with diaminopropanoic acid derivative **106** was achieved by mixing these amino acid derivatives with stoichiometric amounts of TBTU and DiPEA in CH_2Cl_2, to give after 90 minutes and subsequent aqueous work-up, pure dipeptide **107** in 99% yield [Scheme 4.7]. Treatment of **107** with TFA/H_2O gave **108**, *i.e.* Segment B, with an allyl protected carboxylic group. With both key intermediates, Segment A and B, in hand we had expected to complete the synthesis without further delay, since we had already found conditions for oxidizing the α-hydroxy amide unit (Dess-Martin periodinane/*t*-BuOH, see Chapter 5.3) and for subsequent final deprotection of the arginine side chain (TFA/thioanisole).

Unfortunately, the presence of an unprotected hydroxyphenyl group was found to be incompatible with oxidation of the α-hydroxy amide by the Dess-Martin periodinane. Upon treatment of *N*-Boc tyrosine methyl ester with the periodinane, the aromatic signals in the ^{1}H-NMR spectrum rapidly disappeared, suggesting oxidation of the hydroxyphenyl ring into a quinonic structure.
Oxidative cyclisation of *N*-protected tyrosine derivatives by hypervalent iodine species, involving quinonic intermediates, was recently described in detail by Wipf and Kim.[14,15]
From this, it became clear that the hydroxyphenyl group should be protected during the oxidation of the α-hydroxy amide. Consequently, cleavage of the *t*-butyl ether of **107** was not desirable. Thus, we took up the challenge of finding conditions for selective *N*-Boc cleavage in the presence of the aryl *t*-butyl ether in dipeptide **107**, rather then revising the protecting group strategy for Segment B.

Scheme 4.7. Synthesis and *O,N*-deprotection of dipeptide **107**.



106 **100**

a) TBTU/DiPEA, CH_2Cl_2, 90 min; 99%; b) TFA/H_2O, 45 min, 100%.

Selective *N*-Boc deprotection was studied employing **35** (see Scheme 4.5) as a model compound. Treatment of **35** with acids at different concentrations and solvents, *e.g.* HCl/Et_2O, TFA/H_2O (CH_2Cl_2), *p*-TsOH/CH_2Cl_2(Et_2O),[16] HCO_2H/H_2O,[17] aqueous H_2SO_4 (10%)/1,4-dioxane,[18] did not give the desired selectivity. Under some conditions the *t*-butyl ether was even more labile than the Boc group.

The extreme sensitivity of an *O*-(*t*-butyl) tyrosine derivative upon careful treatment with HCl/ethyl acetate (1 M, 500 mol%) was also demonstrated nicely in a recent paper on the selective cleavage of *N*-Boc groups in the presence of other acid-labile protection groups, such as *t*-butyl esters, aliphatic *t*-butyl ethers, *S*-Boc groups, and *S*-trityl ethers.[19]

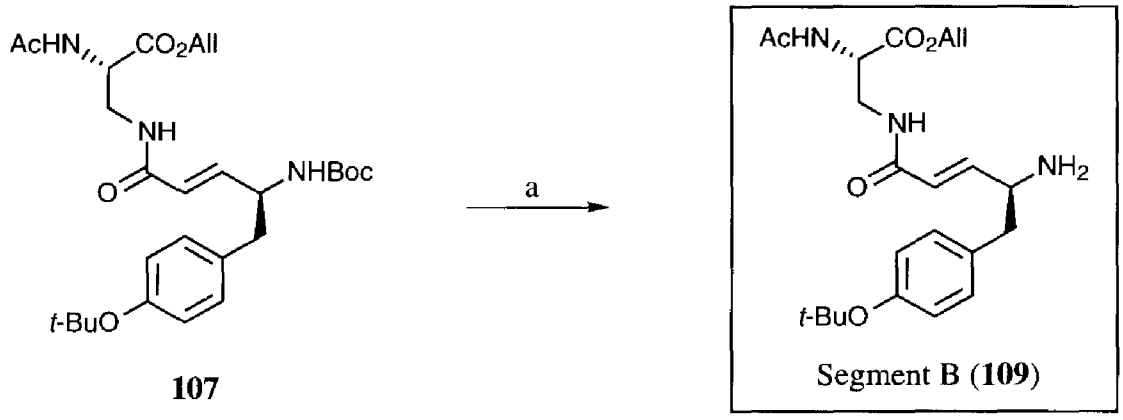
There is only a limited number of methods available for removal of a Boc-group avoiding the use of acidic conditions.[20] Trimethylsilyl trifluoromethanesulfonate (TMS-triflate) and *t*-butyldimethylsilyl trifluoromethanesulfonate (TBDMS-triflate) are known to effect trans-esterfication of Boc groups to give TMS- and TBDMS-carbamates.[21,22,23] These silyl carbamates may be reacted with electrophiles to give alkyl carbamates, or just hydrolyzed (and decarboxylated) to yield a free amine. When only deprotection is required, the use of TMS-triflate is superior to TBDMS-triflate as TMS-carbamates are more readily cleaved than TBDMS-carbamates; however, TBDMS-triflate appears to be more selective.[23] Ohfune *et al.* showed that transformation of a *N*-Boc group to a *t*-butyldimethylsilyl carbamate employing TBDMS-triflate/2,6-lutidine is compatible with the presence of other acid sensitive groups, *e.g.* silyl ethers, acetonides, THP ethers, and *t*-butyl esters.[22,23]

On the basis of this information we treated **35** with TMS-triflate/2,6-lutidine in dry CH_2Cl_2. This indeed resulted in a clean and selective cleavage of the *N*-Boc group without effecting the aryl *t*-butyl ether. Furthermore, it was found that the stoichiometry of the reaction was dependent on the number of exchangeable hydrogen atoms.

Whereas deprotection of *N*-Boc proline required treatment with 1.1 equiv TMS-triflate/1.5 equiv 2,6-lutidine, for complete *N*-Boc cleavage in **35** 2.1 equiv TMS-triflate/2.5 equiv 2,6-lutidine was required. Treatment of *N*-Boc, O-(*t*-butyl)tyrosine *t*-butyl ester with 2.1 equiv TMS-triflate/2.5 equiv 2,6-lutidine resulted in a selective cleavage of the *N*-Boc group, whereas upon treatment with 3.1 equiv TMS-triflate/3.4 equiv 2,6-lutidine also the *t*-butyl ester was cleaved.

Application of these conditions (4.1 equiv TMS-triflate/5.0 equiv 2,6-lutidine) to dipeptide **107** effected selective cleavage of the *N*-Boc group to give, after aqueous work-up, amine **109** in quantitative yield, only slightly contaminated with 2,6-lutidine [Scheme 4.8].

Scheme 4.8. Selective *N*-Boc cleavage in **107** with TMS-triflate/2,6-lutidine, to give Segment B. (**109**).



107

a) TMS-triflate (4.1 equiv)/2,6-lutidine (5 equiv), CH_2Cl_2, 0 °C→rt, 2 h, 100%.

Thus, the desired orthogonality of the *N*-Boc group and the aryl *t*-butyl ether was achieved, and protected Segment B (**109**) was now ready to be coupled with protected Segment A (**97**) [Chapter Five]. Although we originally had not incorporated the presence of the *t*-butyl ether in our synthetic strategy, this acid-labile protecting group should not pose any problem, since it will be cleaved under the same conditions as employed for final deprotection of the bis-Boc protected guanidino unit in the cyclic peptide.

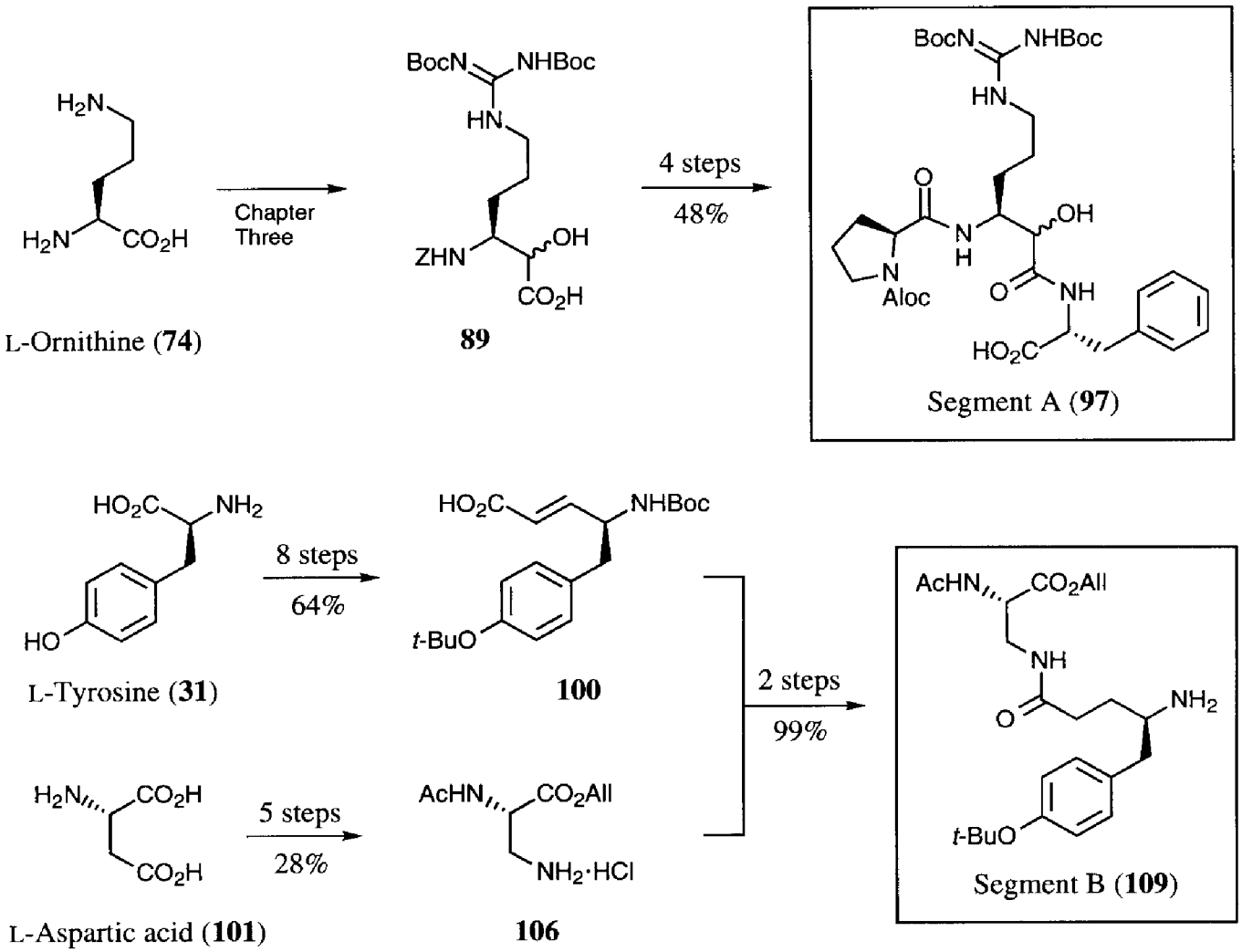
4.4. Conclusions

In this chapter, the synthesis of the two key intermediates, Segment A (**97**) and Segment B (**109**) is described [Scheme 4.9]. Of the two α-hydroxy-β-homoarginine building blocks which we prepared, *i.e.* **73** and **89**, the latter was successfully elaborated into tripeptide **97**. The other β-homoarginine derivative (**73**) failed to give a tripeptide, as conditions employed for the deprotection of the Teoc group were found to be incompatible with the presence of a benzyl ester.

For the synthesis of Segment B (**109**), the constituent amino acids were successfully prepared from common α-amino acids, *viz.* L-tyrosine (**31**) and L-aspartic acid (**101**) [Scheme 4.9]. Fortunately, we were able to selectively remove the *N*-Boc group of **107** to yield **109** without affecting the very sensitive aryl *t*-butyl ether. The presence of this *t*-butyl ether protecting group was found to be necessary to prevent severe side-reactions during oxidation of the α-hydroxy-β-homoarginine unit.

As the two key intermediates **97** and **109** are provided with a set of protecting groups, the properties of which allow oxidation of the hydroxy amide unit and ultimate deprotection, the synthesis of the target molecule now seemed to be within reach...

Scheme 4.9. The two key intermediates, Segment A (**97**) and Segment B (**109**).



4.5. Experimental

Detailed general experimental information is given in Section 3.6.

Benzyl 2(*R*)-[[2(*R,S*)-hydroxy-3(*S*)-[[2-(trimethylsilyl)ethoxycarbonyl]amino]-6-[[imino[(1-adamantoxycarbonyl)amino]methyl](1-adamantoxycarbonyl)amino]hexanoyl]amino]-3-phenyl-propanoate (93)

To a stirred solution of acid **73** (2.54 g, 3.6 mmol) and HOBt (0.95 g, 7.00 mmol) in THF (75 mL) at 0 °C was added DCC (0.80 g, 3.88 mmol). After 40 min, a solution of D-phenylalanine benzyl ester hydrochloride (1.11 g, 3.80 mmol) and TEA (0.53 mL, 3.80 mmol) in THF (50 mL) was added. After another 60 min the ice-bath was removed and the reaction mixture was stirred for 16 h. Subsequently, the reaction mixture was filtrated, diluted with CH_2Cl_2 (250 mL), and washed sequentially with H_2O

(2x), aqueous $NaHCO_3$ (5%, 3x), H_2O (100 mL), aqueous $KHSO_4$ (6%, 3x), H_2O and brine (100 mL), dried (Na_2SO_4), filtrated and concentrated *in vacuo* to give, after column chromatography (EtOAc/PE, 3:2), dipeptide **93** as a white foam (2.12 g; 63%).

^{1}H-NMR ($CDCl_3$): 0.05 (s, 9H, $Si(CH_3)_3$), 1.02 (m, 2H, CH_2Si), 1.40-2.05 (m, 16H, hArg γ-, and δ-H CH_2 Adoc), 2.06-2.33 (m, 18H, CH_2 Adoc and CH Adoc), 3.09 (m, 2H, Phe β-H), 3.64-4.05 (m, 2H, hArg ε-H), 4.05-4.26 (m, 4H, hArg α- and β-H, $C\underline{H}_2CH_2Si$), 4.98 (m, 1H, Phe α-H), 5.11 and 5.17 (AB-system, *J*=11.7, 2H, $OC\underline{H}_2Ph$), 5.66 (bd, *J*=6.7, 1H, hArg α-OH), 6.34 (bd, *J*=8.0, 1H, hArg β-NH), 7.03-7.13 (m, 2H, Phe aryl), 7.18-7.44 (m, 8H, Phe aryl, $OCH_2\underline{Ph}$), 7.53 (bd, *J*=8.3, 1H, Phe α-NH) and 9.33 (bs, 2H, hArg ω- and ω'-NH). ^{13}C-NMR ($CDCl_3$): –1.07 ($Si(CH_3)_3$), 17.7 (CH_2Si), 24.2 (δ-hArg), 25.8 (γ-hArg), 30.6 (CH, Adoc), 30.8 (CH, Adoc), 35.8 (CH_2, Adoc), 36.1 (CH_2, Adoc), 38.2 (β-Phe), 40.0 (CH_2, Adoc), 41.1 (CH_2, Adoc), 43.8 (ε-hArg), 52.0 (β-hArg), 52.6 (α-Phe), 63.3 ($\underline{C}H_2CH_2Si$), 66.9 ($O\underline{C}H_2Ph$), 74.7 (α-hArg), 79.2 (OC, Adoc), 83.8 (OC, Adoc), 126.8 (aryl), 128.3 (aryl), 128.3 (aryl), 128.4 (aryl), 129.1 (aryl), 135.0 (aryl-1), 135.6 (aryl-1), 154.3 (C(O)O, Adoc), 158.3 (C(O)O, Teoc), 160.5 (C(O)O, Adoc), 162.6 (C=N), 170.8 (C(O)NH) and 171.9 ($\underline{C}(O)OCH_2Ph$).

Methyl 2(*R*)-[[2(*R*,*S*)-hydroxy-3(*S*)-[(phenylmethoxycarbonyl)amino]-6-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]methyl]amino]hexanoyl]amino]-3-phenylpropanoate (94)

To a stirred solution of acid **89** (4.78 g, 8.87 mmol) and HOBt (2.40 g, 17.8 mmol) in CH_2Cl_2 (65 mL) at 0 °C was added DCC (2.01 g, 9.72 mmol). After 45 min, a solution of D-phenylalanine methyl ester hydrochloride (2.10 g, 9.74 mmol) and DiPEA (1.81 mL, 10.4 mmol) in CH_2Cl_2 (25 mL) was added. After another 30 min the ice-bath was removed, and the reaction mixture was stirred for 18 h. Subsequently, the reaction mixture was filtrated, diluted with EtOAc (500 mL), and washed sequentially with H_2O (2x), aqueous $NaHCO_3$ (5%, 3x), H_2O, aqueous $KHSO_4$ (6%, 3x), H_2O and brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo*. The crude product was purified by MPLC (EtOAc/PE, 1:1), to give dipeptide **94** as a white foam (3.81 g; 61.3%).

^{1}H-NMR (major isomer, $CDCl_3$): 1.43 (s, 9H, Boc), 1.47 (s, 9H, Boc), 1.45-1.65 (m, 4H, hArg γ- and δ-H), 3.01 (d, *J*=6.7, 2H, Phe β-H), 3.35 (m, 2H, hArg ε-H), 3.68 (s, 3H, OCH_3), 3.98 (m, 1H, hArg β-H), 4.15 (m, 1H, hArg α-H), 4.73-4.90 (m, 2H, Phe α-H, hArg α-OH), 5.04 (bs, 2H, $OC\underline{H}_2Ph$), 5.73 (bd, *J*=8.3, 1H, hArg β-NH), 7.00-7.09 (m, 2H, Phe aryl), 7.14-7.49 (m, 8H, Phe α-NH and aryl, $OCH_2\underline{Ph}$), 8.33 (bt, *J*=5.4, 1H, hArg ε-NH) and 11.42 (bs, 1H, hArg ω-NH). ^{13}C-NMR (major isomer, $CDCl_3$): 25.7 (δ-hArg), 26.7 (γ-hArg), 27.9 ($OC(\underline{C}H_3)_3$), 28.0 ($OC(\underline{C}H_3)_3$), 38.0 (β-Phe), 40.0 (ε-hArg), 52.1 (OCH_3), 52.6 (α-Phe), 53.3 (β-hArg), 67.8 ($O\underline{C}H_2Ph$), 72.9 (α-hArg), 79.3 ($O\underline{C}(CH_3)_3$), 83.0 ($O\underline{C}(CH_3)_3$), 127.0 (aryl), 127.9 (aryl), 127.9 (aryl), 128.3 (aryl), 128.4 (aryl), 129.0 (aryl), 135.6 (aryl-1), 136.1 (aryl-1), 153.0 (C(O)O, Boc), 156.2 (C=N), 156.7 (C(O)O, Z), 163.1 (C(O)O, Boc), 171.5 (C(O)NH) and 171.8 ($\underline{C}(O)OCH_3$).

Methyl 2(*R*)-[[2(*R*,*S*)-hydroxy-3(*S*)-amino-6-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]methyl]amino]hexanoyl]amino]-3-phenylpropanoate (95)

A solution of **94** (6.30 g, 9.00 mmol) in MeOH (25 mL) was stirred vigorously with Pd/C (5%, 0.92 g) in a H_2 atmosphere during 6 h. Filtration and evaporation of the solvent *in vacuo* yielded **95** as a white foam (5.28 g; 100%).

^{1}H-NMR (major isomer, $CDCl_3$): 1.48 (s, 18H, 2x Boc), 1.48-1.80 (m, 4H, hArg α-OH, β-NH_2, γ- and

δ-H), 2.96 (dd, *J*=13.7 and 7.4, 1H, Phe β-H), 3.08-3.20 (m, 2H, Phe β-H, hArg β-H), 3.37 (m, 2H, hArg ε-H), 3.70 (s, 3H, OCH_3), 3.79 (d, *J*=2.4, 1H, hArg α-H), 4.91 (m, 1H, Phe α-H), 7.02-7.18 (m, 2H, Phe aryl), 7.18-7.31 (m, 3H, Phe aryl), 7.49 (bd *J*=8.4, 1H, Phe α-NH), 8.32 (bt, *J*=5.4, 1H, hArg ε-NH) and 11.44 (bs, 1H, hArg ω-NH). ^{13}C-NMR (major isomer, $CDCl_3$): 25.9 (δ-hArg), 27.9 ($OC(\underline{C}H_3)_3$), 28.1 ($OC(\underline{C}H_3)_3$), 29.8 (γ-hArg), 38.1 (β-Phe), 40.3 (ε-hArg), 52.2 (OCH_3), 52.3 (β-hArg), 52.5 (α-Phe), 72.7 (α-hArg), 79.1 ($O\underline{C}(CH_3)_3$), 83.0 ($O\underline{C}(CH_3)_3$), 126.9 (aryl-4), 128.4 (aryl-2), 129.0 (aryl-3), 135.9 (aryl-3), 153.1 (C(O)O, Boc), 156.0 (C=N), 163.0 (C(O)O, Boc), 171.6 (C(O)NH) and 172.7 ($\underline{C}(O)OCH_3$).

Methyl 2(*R*)-[[2-(*R,S*)-hydroxy-3(*S*)-[[[1-(allyloxycarbonyl)pyrrolidin-2(*S*)-yl]carbonyl]-amino]-6-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]methyl]amino]hexano-yl]amino]-3-phenylpropanoate (96)

To stirred solution of *N*-Aloc proline (1.67 g, 8.38 mmol) and **95** (4.31 g, 7.62 mmol) in CH_2Cl_2 (50 mL) was added DiPEA (1.40 mL, 8.04 mmol) and TBTU (2.57 g, 8.00 mmol). After 2.5 h the reaction mixture was diluted with EtOAc (250 mL) and washed sequentially with H_2O (4x), aqueous $NaHCO_3$ (5%, 3x), H_2O, aqueous $KHSO_4$ (6%, 3x), H_2O and brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo* to give, after purification by MPLC (EtOAc/PE/EtOH, 60:40:2), tripeptide **96** as a white foam (4.50 g; 79.1%).

^{1}H-NMR (major isomer, $CDCl_3$, 400.1 MHz): 1.49 (s, 18H, 2x Boc), 1.45-1.70 (m, 5H, hArg γ- and δ-H, Pro γ-H), 1.75-1.93 (m, 2H, Pro β- and γ-H), 2.07 (m, 1H, Pro β-H), 3.10 (d, *J*=6.6, 2H, Phe β-H), 3.33 (m, 1H, hArg ε-H), 3.39-3.52 (m, 2H, hArg ε-H, Pro δ-H), 3.57 (m, 1H, Pro δ-H), 3.70 (s, 3H, OCH_3), 4.04-4.16 (m, 1H, hArg α- and β-H), 4.25 (m, 1H, Pro α-H), 4.55 (m, 2H, $OC\underline{H}_2CH=CH_2$), 4.78-4.95 (m, 2H, Phe α-H, hArg α-OH), 5.24 (m, 2H, $OCH_2CH=C\underline{H}_2$), 5.79 (m, 1H, $OCH_2-C\underline{H}=CH_2$), 7.10-7.20 (m, 2H, Phe aryl), 7.20-7.34 (m, 5H, Phe aryl and α-NH, hArg β-NH), 8.34 (bt, *J*=5.0, 1H, hArg ε-NH) and 11.49 (bs, 1H, hArg ω-NH). ^{13}C-NMR (major isomer, $CDCl_3$, 100.6 MHz): 23.7 (δ-hArg), 24.5 (γ-hArg), 28.1 ($OC(\underline{C}H_3)_3$), 28.3 ($OC(\underline{C}H_3)_3$), 29.7 (γ-Pro), 31.4 (β-Pro), 38.1 (β-Phe), 40.2 (ε-hArg), 47.3 (δ-Pro, b), 52.3 (OCH_3), 52.8 (β-hArg), 53.0 (α-Phe), 61.0 (α-Pro), 66.3 ($O\underline{C}H_2CH=CH_2$), 73.1 (α-hArg), 79.3 ($O\underline{C}(CH_3)_3$), 83.1 ($O\underline{C}(CH_3)_3$), 117.7 ($OCH_2CH=\underline{C}H_2$), 125.5 (aryl-4), 127.1 (aryl-2), 128.6 (aryl-3), 132.7 ($OCH_2\underline{C}H=CH_2$), 135.9 (aryl-1), 153.2 (C(O)O, Boc), 155.6 (C(O)O, Aloc), 156.3 (C=N), 163.4 (C(O)O, Boc), 171.7 (C(O)NH, hArg-Pro), 171.8 ($\underline{C}(O)OCH_3$) and 173.3 (C(O)NH, Pro-hArg).

2(*R*)-[[2(*R,S*)-Hydroxy-3(*S*)-[[[1-(allyloxycarbonyl)pyrrolidin-2(*S*)-yl]carbonyl]amino]-6-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]methyl]amino]hexanoyl]amino]-3-phenylpropanoic acid (97)

To a vigorously stirred solution of **96** (4.30 g 5.76 mmol) in THF/MeOH/H_2O (187 mL, 3.6:1:1) was added thoroughly powdered $LiOH\cdot H_2O$ (727 mg, 17.3 mmol). After 12 min, aqueous $KHSO_4$ (6%, 40 mL) was added and the organic solvents were evaporated under reduced pressure. The turbid residue was diluted with H_2O (100 mL), acidified to pH≈ 2.5 with aqueous $KHSO_4$ (6%), and extracted with EtOAc (3x 80 mL). The combined organic extracts were washed with H_2O and brine, dried (Na_2SO_4), filtrated, and concentrated under reduced pressure, to yield pure **97** as a white foam (4.21 g; 99.7%).

^{1}H-NMR ($CDCl_3$): 1.47 (s, 9H, Boc), 1.49 (s, 9H, Boc), 1.47-2.08 (m, 8H, hArg γ-, δ-H and Pro β- and γ-H), 2.93-3.60 (m, 6H, hArg ε-H, Phe β-H, Pro δ-H), 3.77-4.26 (m, 3H, hArg α- and β-H, Pro δ-H), 4.37-4.83 (m, 3H, Phe β-H, $OC\underline{H}_2CH=CH_2$), 5.23 (m, 2H, $OCH_2CH=C\underline{H}_2$), 5.86 (m, 1H, OCH_2-

C<u>H</u>=CH_2), 7.12-7.37 (m, 6H, Phe aryl, hArg β-NH), 7.40 (bd, *J*=8.3, 1H, hArg ε-NH) and 8.42 (bs, 1H, Phe α-NH).

Methyl 2(*S*)-[(*tert*-butyloxycarbonyl)amino]-3-[(4-(*tert*-butyloxy)phenyl]propanoate (35)

Prepared in a five step sequence from L-tyrosine using literature procedures[5] to give **35**, after purification by MPLC (EtOAc/PE, 3:1) as a colourless solid in an overall yield of 69.6%; mp 99-100 °C, $[\alpha]_D^{30}$ + 46.5° (*c*=1, $CHCl_3$).

^{1}H-NMR ($CDCl_3$): 1.30 (s, 9H, *t*-Bu), 1.39 (s, 9H, Boc), 3.00 (m, 2H, β-H), 3.68 (s, 3H, OCH_3), 4.53 (m, 1H, α-H), 4.98 (bd, *J*=8.3, 1H, α-NH), 6.90 and 7.00 (AB-system, *J*=9.2, 4H, aryl). ^{13}C-NMR ($CDCl_3$): 28.0 ($OC(\underline{C}H_3)_3$), 28.6 ($C(O)OC(\underline{C}H_3)_3$), 37.4 (β), 51.8 ($OCH_3$), 54.3 (α), 78.0 ($O\underline{C}(CH_3)_3$), 79.4 ($C(O)O\underline{C}(CH_3)_3$), 123.9 (aryl-3), 129.4 (aryl-2). 130.7 (aryl-1), 154.1 (aryl-4), 154.8 (C(O)O, Boc) and 172.2 ($\underline{C}(O)OCH_3$). FAB-HRMS: calcd for $[C_{19}H_{29}NO_5 + H]^+$ 352.2123, found 352.2135.

2(*S*)-[(*tert*-Butyloxycarbonyl)amino]-3-[4-(*tert*-butyloxy)phenyl]propanal (98)

To a stirred solution of **35** (8.79 g, 25.0 mmol) in CH_2Cl_2 (250 mL) at –70 °C was added DiBAH (1 N in hexanes, 62.5 mL, 62.5 mmol) in 15 min, after which the reaction was usually complete (monitored by TLC). Subsequently, a solution of EtOH/36% HCl_{aq} (9:1, 6.0 mL) was added slowly (the temperature was kept below –65 °C). The reaction mixture was added to a vigorously stirred solution of HCl_{aq} (1 N, 400 mL) at 0 °C. The layers were separated, and the aqueous layer was extracted with ice-cold CH_2Cl_2 (2x 150 mL). The combined organic layers were washed with ice-cold HCl_{aq} (1 N, 2x 150 mL), ice-cold H_2O (2x 150 mL) and ice-cold brine, dried (Na_2SO_4), and concentrated *in vacuo* at ambient temperature, to give aldehyde **98** as a colourless oil (8.04 g; 100% crude yield).

^{1}H-NMR ($CDCl_3$): 1.31 (s, 9H, *t*-Bu), 1.42 (s, 9H, Boc), 3.07 (bd, *J*=6.7, 2H, β-H), 4.43 (m, 1H, α-H), 5.06 (bd, *J*=8.1, 1H, α-NH), 6.91 and 7.01 (AB-system, *J*=9.2, 4H, aryl) and 9.61 (s, 1H, C(O)H).

Ethyl 4(*S*)-[(*tert*-butyloxycarbonyl)amino]-5-[4-(*tert*-butyloxy)phenyl]pent-2(*E*)-enoate (99)

A dispersion of NaH (60% in mineral oil, 2.00 g, 50.0 mmol) was washed with Et_2O (2x). THF (130 mL) was added. The stirred suspension was cooled with an ice-bath, and triethyl phosphonoacetate (10.5 mL, 52.5 mmol) was carefully added (H_2 evolution!). Subsequently, the reaction mixture was stirred at room temperature for 10 min. The resulting solution was placed again in an ice-bath, and added dropwise to a solution of **98** (8.04 g, max 25.0 mmol) in THF (200 mL) at –50 °C. After an additional 30 min at –50 °C, the reaction mixture was warmed to 10 °C in 2.5 h. The resulting yellow solution was added to a vigorously stirred mixture of aqueous $NaHCO_3$ (5%)/EtOAc (500 mL, 2:3), the layers were separated, and the aqueous layer was extracted with EtOAc (2x 150 mL). The combined organic layers were washed with H_2O (2x) and brine, dried (Na_2SO_4), filtrated, and concentrated *in vacuo*, to give, after purification by column chromatography (EtOAc/PE, 1:4), **99** as a slightly yellowish oil (9.09 g, 93.7%).

^{1}H-NMR ($CDCl_3$): 1.25 (t, *J*=6.7, 3H, $OCH_2C\underline{H}_3$), 1.30 (s, 9H, *t*-Bu), 1.39 (s, 9H, Boc), 2.80 (m, 2H, δ-H), 4.16 (q, *J*=6.7, 2H, $OC\underline{H}_2CH_3$), 4.39-4.62 (m, 2H, γ-H and γ-NH), 5.80 (dd, *J*=16.7 and 1.5, 1H, α-H), 6.67 (dd, *J*=16.7 and 5.0, 1H, β-H), 6.90 and 7.02 (AB-system, *J*=9.2, 4H, aryl). FAB-HRMS: calcd for $[C_{22}H_{33}NO_5 + H]^+$ 392.2437, found 392.2460.

4(*S*)-[(*tert*-Butyloxycarbonyl)amino]-5-[4-(*tert*-butyloxy)phenyl]pent-2(*E*)-enoic acid (100)

To stirred solution of **99** (9.00 g, 23.0 mmol) in 1,4-dioxane/H_2O (390 mL, 35:4) was added aqueous NaOH (1 N, 23.0 mL) over a period of 2 h, as to maintain the pH at approx 12. The reaction mixture was stirred overnight (the reaction was complete as checked by TLC), acidified to pH≈ 2.5 (with

aqueous 6% $KHSO_4$), diluted with H_2O (100 mL), and extracted with EtOAc (3x 250 mL). The combined organic extracts were washed with H_2O and brine, dried (Na_2SO_4), filtrated, and concentrated under reduced pressure to give, after purification by column chromatography ($CHCl_3$/ MeOH, 98:2), acid **100** as a colourless crystalline solid (8.15 g, 97.5%). The crude product could also be crystallized from $CHCl_3$/hexane; mp 133-135 °C (dec.), $[\alpha]_D^{30}$ + 9.2° (c=1, $CHCl_3$).

^{1}H-NMR ($CDCl_3$, 400.1 MHz): 1.31 (s, 9H, *t*-Bu), 1.39 (s, 9H, Boc), 2.84 (m, 2H, δ-H), 4.46-4.70 (m, 2H, γ-H, γ-NH), 5.83 (dd, J=15.7 and 1.6, 1H, α-H), 6.94 and 7.02 (AB-system, J=8.3, 4H, aryl) and 6.99 (dd, J=15.7 and 5.1, 1H, β-H). ^{13}C-NMR ($CDCl_3$): 28.1 ($OC(\underline{C}H_3)_3$), 28.6 ($C(O)OC(\underline{C}H_3)_3$), 39.9 (δ), 52.3 (γ), 77.3 ($O\underline{C}(CH_3)_3$), 78.3 ($C(O)O\underline{C}(CH_3)_3$), 120.3 (α), 124.1 (aryl-3), 129.6 (aryl-2). 131.0 (aryl-1), 150.0 (β), 154.1 (aryl-4), 154.9 (C(O)O, Boc) and 170.7 (C(O)OH). FAB-HRMS: calcd for $[C_{20}H_{29}NO_5 + H]^+$ 364.2124, found 364.2139.

2(*S*),3-Diaminopropanoic acid monohydrochloride (102)

L-Aspartic acid (**101**) was converted to L-2,3-diaminopropanoic acid hydrochloride according to the method of Rao *et al.*,[12] to give **102** as off-white needles in 58.3 % yield; $[\alpha]_D^{30}$ + 24.8° (c=2, 0.5 N HCl), [lit $[\alpha]_D^{27}$+ 25.2° (c=2, 0.5 N HCl)].

^{1}H-NMR (6% DCl in D_2O): 3.50 (d, J=7.0, 2H, β-H) and 3.99 (t, J=7.0, 1H, α-H).

Allyl 2(*S*),3-diaminopropanoate bis(*p*-toluenesulfonate) (103)

A stirred suspension of **102** (14.1 g, 100 mmol) and *p*-TsOH·H_2O (47.1 g, 247 mmol) in allyl alcohol/ benzene (270 mL, 17:10) was refluxed in a reaction flask equipped with a Dean-Stark trap and a drying tube ($CaCl_2$). After 2 h, a suspension of *p*-TsOH·H_2O (7.50 g, 40.0 mmol) in allyl alcohol (8.0 mL) was added; this addition was repeated three times (at 4, 6 and 8 h). After the last addition, the reaction mixture (which had become clear by now) was refluxed for an additional 4 h, cooled to 45 °C, and added while stirring to ice-cold Et_2O (1 L). The precipitate was collected by filtration, dried *in vacuo* at 40 °C and, subsequently, in a desiccator containing P_2O_5. The crude product was crystallized from MeOH/EtOAc to give allyl ester **103** as colourless needles (37.7 g; 77.2%); mp 159-162 °C (dec.), $[\alpha]_D^{30}$ + 21.3° (c=1, MeOH).

^{1}H-NMR (DMSO-d_6): 2.38 (s, 6H, CH_3 *p*-TsOH), 3.25 (m, 2H, β-H), 4.35 (bt, J=6.7, 1H, α-H), 4.70 (m, 2H, $OC\underline{H}_2CH=CH_2$), 5.36 (m, 2H, $OCH_2CH=C\underline{H}_2$), 5.91 (m, 1H, $OCH_2C\underline{H}=CH_2$), 7.11 and 7.49 (AB-system, J=8.0, 8H, aryl *p*-TsOH), 8.10 (bs, 3H, NH_3^+) and 8.52 (s, 3H, NH_3^+). FAB-HRMS: calcd for $[C_6H_{12}N_2O_6 + H]^+$ 145.0977, found 145.0936.

Allyl 2(*S*)-amino-3-[(*tert*-butyloxycarbonyl)amino]propanoate (104)

A stirred solution of **103** (24.1 g, 50.5 mmol) in CH_2Cl_2 (1.5 L) at –64 °C, was treated with TEA (28.1 mL, 202 mmol) and Boc_2O (11.5 g, 52.7 mmol) in CH_2Cl_2 (100 mL) and worked-up as described by Egbertson *et al.*,[13] to give a yellow oil (mixture of regioisomers, 5.90 g, max 47.9%). The crude product was purified by MPLC (EtOAc/MeOH, 95:5), to give **104** as a slightly coloured oil (1.40 g; 11.3%).

^{1}H-NMR ($CDCl_3$): 1.41 (s, 9H, Boc), 2.28 (bs, 2H, NH_2), 3.28 (m, 1H, β-H), 3.50 (m, 1H, β-H), 4.59 (t, J=5.8, 1H, α-H), 4.60 (m, 2H, $OC\underline{H}_2CH=CH_2$), 4.99 (m, 1H, β-NH), 5.28 (m, 2H, $OCH_2CH=C\underline{H}_2$) and 5.89 (m, 1H, $OCH_2C\underline{H}=CH_2$).

Allyl 2(*S*)-acetylamino-3-[(*tert*-butyloxycarbonyl)amino]propanoate (105)

*From **104**;* To a stirred solution of **104** (1.40 g, 5.73 mmol) and pyridine (1.38 mL, 17.1 mmol) in MeCN (33 mL) at 0 °C, was added acetyl chloride (0.54 mL, 6.20 mmol, freshly distilled). The

reaction mixture was allowed to warm to room temperature, and was stirred for 15 h. The reaction mixture was diluted with EtOAc (200 mL), washed with aqueous $KHSO_4$ (6%, 4x), H_2O and brine, dried (Na_2SO_4), filtrated, and concentrated *in vacuo* to give, after purification by centrifugal chromatography (CH_2Cl_2/THF, 95:5), **105** as a colourless oil (0.87 g; 53.2%, 6.0% from **103**).

*From **103** by a two-step, one-pot procedure;* TEA (14.1 mL, 100 mmol) was slowly added to a stirred suspension of **103** (12.2 g, 25.0 mmol) in CH_2Cl_2 (750 mL) at –62 °C. To the resulting solution, Boc_2O (5.42 g, 24.8 mmol) in CH_2Cl_2 (40 mL) was added over a period of 2 h. Subsequently, the reaction mixture was cooled in an ice-bath, and stirring was continued for 2 h, after which acetyl chloride (7.85 mL, 100 mmol, freshly distilled) was added dropwise. The reaction mixture was stirred overnight (temperature < 20 °C), concentrated under reduced pressure at 20 °C to *ca* 250 mL, and poured onto saturated aqueous $NaHCO_3$ (250 mL). The layers were separated, and the organic layer was sequentially washed with, H_2O, aqueous $KHSO_4$ (6%, 3x), H_2O and brine, dried (Na_2SO_4), filtrated, and concentrated *in vacuo* to give, after purification by MPLC (CH_2Cl_2/EtOAc, 84:16), **105** as a colourless oil which solidified upon standing (4.60 g; 64.3% from **97**); mp 71-72 °C, $[\alpha]_D^{30}$ + 11.8° (*c*=1, $CHCl_3$).

^{1}H-NMR ($CDCl_3$): 1.41 (s, 9H, Boc), 2.01 (s, 3H, Ac), 3.53 (t, *J*=5.8, 2H, β-H), 4.53-4.72 (m, 3H, α-H and $OC\underline{H}_2CH{=}CH_2$), 4.90 (bt, *J*=5.7, 1H, β-NH), 5.30 (m, 2H, $OCH_2CH{=}C\underline{H}_2$), 5.90 (m, 1H, $OCH_2C\underline{H}{=}CH_2$) and 6.70 (bd, *J*=7.0, 1H, α-NH). ^{13}C-NMR ($CDCl_3$): 23.1 ($C(O)\underline{C}H_3$), 28.2 ($OC(C\underline{H}_3)_3$), 42.1 (β), 53.9 (α), 66.3 ($OC\underline{H}_2CH{=}CH_2$), 80.1 ($O\underline{C}(CH_3)_3$), 119.0 ($OCH_2CH{=}C\underline{H}_2$), 131.4 ($OCH_2\underline{C}H{=}CH_2$), 156.7 (C(O)O, Boc), 170.1 ($\underline{C}(O)CH_3$) and 170.3 ($\underline{C}(O)OAll$). FAB-HRMS: calcd for $[C_{13}H_{22}N_2O_5 + H]^+$ 287.1607, found 287.1584.

Allyl 2(*S*)-acetylamino-3-aminopropanoate hydrochloride (106)

To a stirred solution of **105** (4.08 g, 14.2 mmol) in Et_2O (50 mL) was added an etheral solution of HCl (4.0 M, 100 mL) of 5 °C. After 40 min the turbid reaction mixture was concentrated under reduced pressure. The residue was triturated with Et_2O, dried under reduced pressure and further dried in a desiccator containing KOH and P_2O_5, to give pure **106** as an amorphous powder (3.18 g; 100 %). Recrystallization from EtOAc/MeOH furnished **106** as colourless needles (2.73 g; 86.4%); mp 142.5-144 °C, $[\alpha]_D^{30}$ – 30.4° (*c*=1, MeOH).

^{1}H-NMR (DMSO-d_6): 1.89 (s, 3H, Ac), 2.98-3.31 (m, 2H, β-H), 4.48-4.67 (m, 3H, α-H and $OC\underline{H}_2$-$CH{=}CH_2$), 5.30 (m, 2H, $OCH_2CH{=}C\underline{H}_2$), 5.89 (m, 1H, $OCH_2C\underline{H}{=}CH_2$), 8.28 (bs, 3H, β-$NH_3^+$) and 8.66 (bd, *J*=8.2, 1H, α-NH). FAB-HRMS: calcd for $[C_8H_{14}N_2O_3 + H]^+$ 187.1083, found 187.1098.

Allyl 2(*S*)-acetylamino-3-[[4(*S*)-[(*tert*-butyloxycarbonyl)amino]-5-[4-(*tert*-butyloxy)phenyl]-pent-2(*E*)-enoyl]amino]propanoate (107)

To a stirred solution of acid **100** (2.00 g, 5.50 mmol) and amine **106** (1.33 g, 5.97 mmol) in CH_2Cl_2 (50 mL), was added DiPEA (2.09 mL, 12.0 mmol) followed by TBTU (1.92 g, 6.00 mmol). The reaction was complete after 1.5 h (checked by TLC). The reaction mixture was diluted with EtOAc (200 mL) and sequentially washed with H_2O (3x), aqueous $NaHCO_3$ (5%, 3x), H_2O, aqueous $KHSO_4$ (6%, 3x), H_2O (2x) and brine, dried (Na_2SO_4), filtrated, and concentrated *in vacuo* to give pure **107** as a colourless glass (2.89 g, 98.8%); mp 134-135 °C, $[\alpha]_D^{30}$ – 49.2° (*c*=1, $CHCl_3$).

^{1}H-NMR ($CDCl_3$): 1.30 (s, 3H, *t*-Bu), 1.39 (s, 9H, Boc), 2.01 (s, 3H, Ac), 2.79 (m, 2H, vTyr δ-H), 3.70 (t, *J*=5.8, 2H, Dpr β-H), 4.44-4.71 (m, 5H, vTyr γ-H and γ-NH, Dpr α-H, $OC\underline{H}_2CH{=}CH_2$), 5.30 (m, 2H, $OCH_2CH{=}C\underline{H}_2$), 5.79 (d, *J*=15.6, 1H, vTyr α-H), 5.90 (m, 1H, $OCH_2C\underline{H}{=}CH_2$), 6.30 (bs, 1H,

Dpr β-NH), 6.79 (dd, *J*=15.6 and 4.9, 1H, vTyr β-H), 6.90 and 7.06 (AB-system, *J*=8.4, 4H, vTyr aryl) and 7.05 (m overlapping, 1H, Dpr α-NH). ^{13}C-NMR ($CDCl_3$): 22.6 (C(O)$\underline{C}H_3$), 28.0 (OC($C\underline{H}_3$)$_3$), 28.5 (C(O)OC($C\underline{H}_3$)$_3$), 40.0 (δ-vTyr), 40.9 (β-Dpr), 52.4 (γ-vTyr), 53.1 (α-Dpr), 66.1 ($OC\underline{H}_2$-CH=CH_2), 78.0 (O$\underline{C}$(CH_3)$_3$), 79.3 (C(O)O$\underline{C}$(CH_3)$_3$), 119.7 ($OCH_2CH=C\underline{H}_2$), 122.6 (α-vTyr), 123.8 (aryl-3), 129.5 (aryl-2), 131.1 ($OCH_2\underline{C}H=CH_2$), 131.4 (aryl-1), 144.0 (β-vTyr), 153.7 (aryl-4), 154.9 (C(O)O, Boc), 166.5 (C(O)NH), 170.0 (C(O)CH_3) and 170.6 ($\underline{C}$(O)OAll). FAB-HRMS: calcd for $[C_{28}H_{41}N_3O_7 + H]^+$ 532.3023, found 532.3042.

Allyl 2(*S*)-acetylamino-3-[[4(*S*)-amino-5-[4-(*tert*-butyloxy)phenyl]pent-2-(*E*)-enoyl]amino]-propanoate (109)

To a vigorously stirred solution of dipeptide **107** (1.06 g, 2.00 mmol) and 2,6-lutidine (1.17 mL, 10.0 mmol) in CH_2Cl_2 (4.0 mL) at 0 °C, was added TMS-triflate (1.60 mL, 8.00 mmol). After 15 min, the ice-bath was removed, and stirring was continued for 95 min. The reaction mixture was placed again in an ice-bath, and ice-cold saturated NH_4Cl_{aq} (16 mL) was added. Subsequently, the quenched reaction mixture was extracted with EtOAc (2x 30 mL). The combined organic extracts were washed with saturated aqueous $NaHCO_3$ and brine, dried (Na_2SO_4), filtrated, and concentrated under reduced pressure to give amine **109** (contaminated with 2,6-lutidine) as an oil (1.00 g, essentially quantitative). ^{1}H-NMR ($CDCl_3$): 1.30 (s, 3H, *t*-Bu), 2.01 (s, 3H, Ac), 2.31 (bs, 2H, vTyr γ-NH_2), 2.60 (dd, *J*=4.9 and 17.9, 1H, vTyr δ-H), 2.88 (dd, *J*=8.4 and 17.9, 1H, vTyr δ-H), 3.61-3.82 (m, 3H, vTyr γ-H, Dpr β-H), 4.58-4.71 (m, 3H, Dpr α-H, $OC\underline{H}_2CH=CH_2$), 5.30 (m, 2H, $OCH_2CH=C\underline{H}_2$), 5.89 (m, 1H, $OCH_2C\underline{H}=CH_2$), 5.92 (dd, *J*=15.4 and 1.5, 1H, vTyr α-H), 6.51 (bs, 1H, Dpr β-NH), 6.72 (dd, *J*=15.5 and 5.1, 1H, vTyr β-H), 6.88 (bs, 1H, Dpr α-NH), 6.92 and 7.08 (AB-system, *J*=8.2, 4H, vTyr aryl).

4.6. References and Notes

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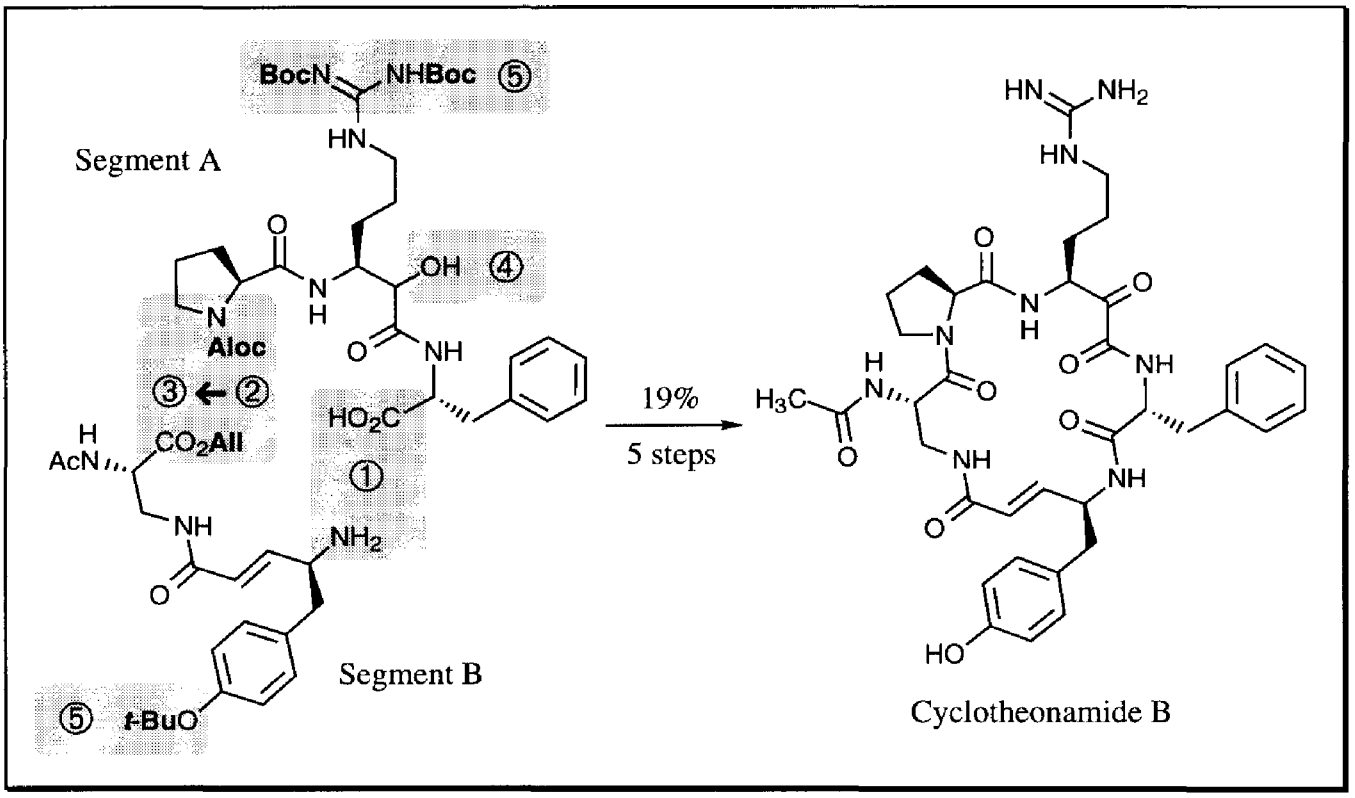
CHAPTER FIVE

Synthesis of Cyclotheonamide B from the Key Intermediates



Abstract

Elaboration of the two key intermediates, Segment A (**97**) and Segment B (**109**), in a five-step fashion *i.e.*: ① fragment condensation, ② *O,N*-deprotection, ③ cyclization, ④ oxidation, and finally, ⑤ deprotection, proceeded smoothly and gave Cyclotheonamide B in 19% overall yield from Segment A.

Synthetic Cyclotheonamide B was identical in all respects (NMR, FAB-MS, optical rotation and bioassay) to the natural product.

5.1. Introduction

In this Chapter the synthesis of Cyclotheonamide B, through elaboration of the two protected key intermediates Segment A (**97**) and Segment B (**109**), is described. Since the viability of the protecting group regime on the two intermediates had been verified to secure late stage oxidation and final deprotection, the stage was set for the construction of the macrocyclic ring [Section 5.3].
We foresaw two problems. As discussed in Chapter 2.3, activation of the carboxyl group of Segment A (**97**) upon fragment condensation might lead to δ-lacton formation through intramolecular nucleophilic attack of the unprotected hydroxyl group of the β-homoarginine residue. Protection of this hydroxyl group might then again be considered. Furthermore, the lower reactivity of the proline nitrogen atom (as compared to primary nitrogen atoms of other amino acids) might lower the yield of the cyclization step. However, we anticipated that by the use of a modern and powerful coupling reagent, such as 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) [Chapter 4.2.2], fragment condensation and ring closure would be viable and that these two potential problems could be overcome.

5.2. Segment Coupling and Macrocyclization

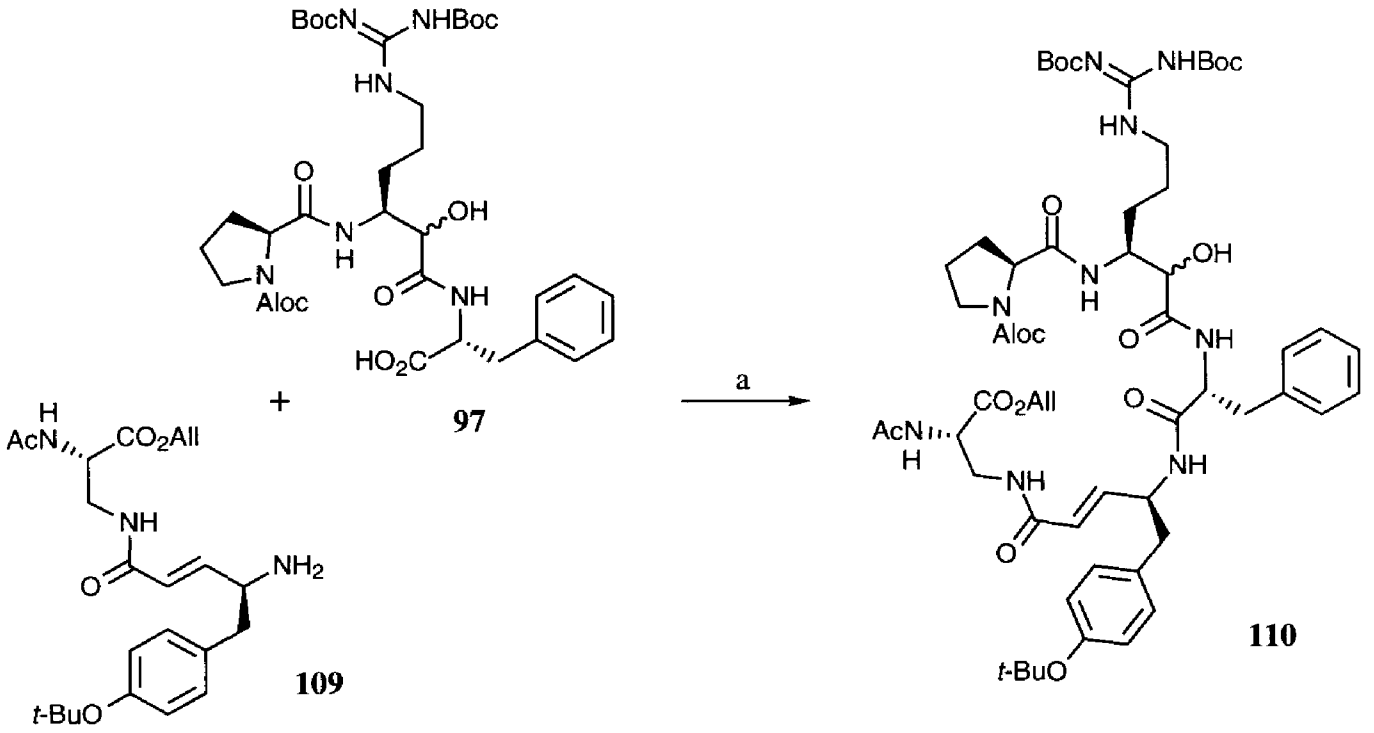
5.2.1. Fragment condensation

The TBTU-coupling method [Chapter 4.2.2] was used to effect the coupling of Segment A (**97**) with Segment B (**109**) [Scheme 5.1]. By employing this method the possible δ-lacton formation is suppressed since the reagent is added to the *mixture* of both reaction partners. Thus, the carboxyl group of **97** is activated in the presence of the primary amino group of **109** which has a higher nucleophilicity than the secondary alcohol of **97**.
The superiority of the coupling reagent was demonstrated once more: after a reaction time of just 2-3 hours the protected linear pentapeptide **110** was isolated in 85 % yield after purification by chromatography.

5.2.2. *O,N*-Deprotection

Before cyclization can take place, the *C*-terminal allyl group and the *N*-terminal Aloc group of **110** have to be removed. In the Pd(0)-catalyzed deprotection of allyl esters and allyl carbamates, the allyl group is transferred, *via* a π-allyl–Pd complex, to an acceptor.
A large series of nucleophilic allyl acceptors has been developed, *e.g.* secondary amines, carboxylic acids, and CH-acids. Deprotection of a simple N^{α}-Aloc-β-homoarginine derivative using the CH-acid dimedone (pK_a= 5.2) as an allyl acceptor is described in Chapter 3.2.2. Also for the deprotection of pentapeptide **110** we preferred to first study the employment of CH-acids, because of their relative inertness. Both dimedone[1] and dimethylbarbituric acid[2] (pK_a= 4.7) were tested. Although de-allylation proceeded smoothly, attempts to remove the excess of acceptor from the reaction mixture by extraction with aqueous $NaHCO_3$ were unsuccessful.

Scheme 5.1. Coupling of Segment A (**97**) and Segment B (**109**).



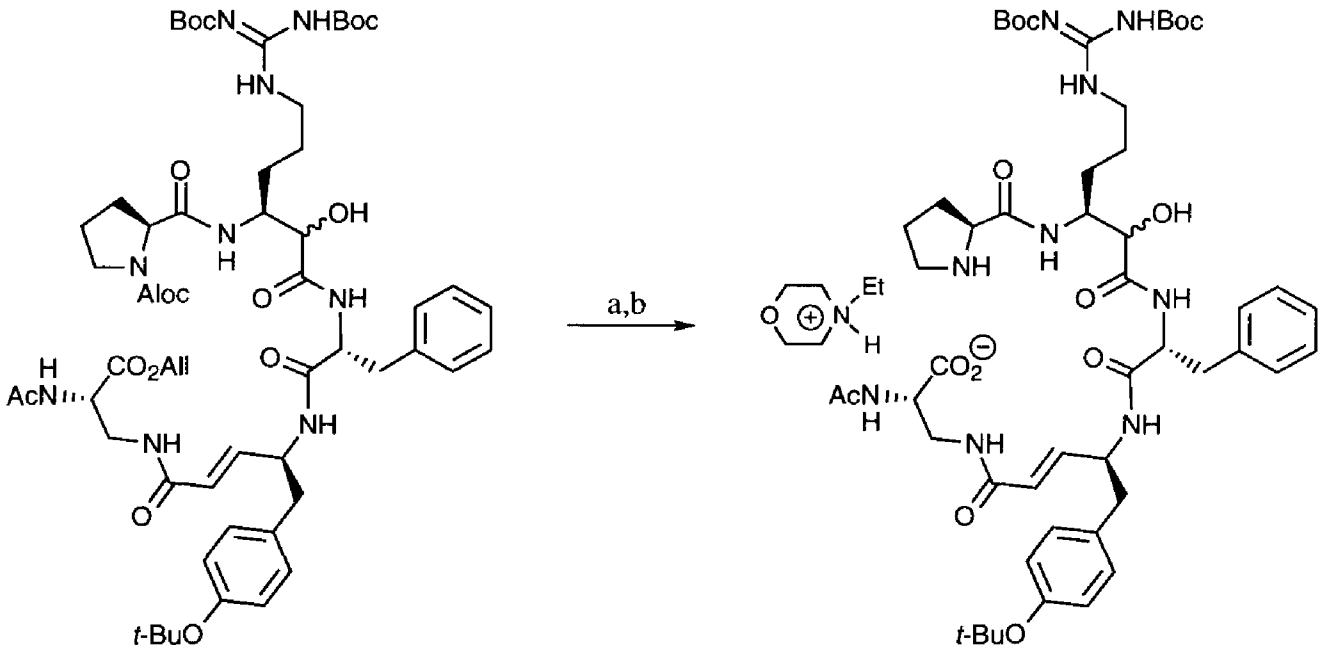
a) TBTU/DiPEA, CH_2Cl_2, 3 h, 85%.

Dimethyl malonate, a more volatile CH-acid (hence removable by evaporation instead of extraction), has been successfully used as an allyl acceptor in de-allylation of *N*-Aloc protected carbohydrates.[3] However, the effectiveness of dimethyl malonate (pK_a= 11) in the model compound Aloc-Pro–Ala-OMe was disappointing: treatment with 5 mol% $Pd(PPh_3)_4$ in neat dimethyl malonate resulted in extensive formation of the *N*-allyl proline derivative. We therefore took resort to a secondary amine. Morpholine[4] appeared to work nicely (50 equiv, THF, 7 mol% $Pd(PPh_3)_4$, rt, 45 min). After repeated evaporation to remove *N*-allyl morpholine and the excess of morpholine, the ^{1}H-NMR spectrum still showed morpholine signals, probably due to the formation of an ammonium-salt with the liberated *C*-terminal carboxyl group. A procedure to remove the residual morpholine was devised, as we were concerned that this morpholine, being a secondary amine, would compete with the proline nitrogen during the cyclization step.

In a small-scale experiment the crude reaction product, obtained after evaporation of THF, was dissolved in ice-cold aqueous $NaHSO_4$ (2%) and extracted with THF to remove the catalyst. Subsequently, the aqueous phase was saturated with NaCl and extracted with ice-cold THF. The organic phase was dried and evaporated to give the morpholine-free pentapeptide. However, on a larger scale the acid-labile *t*-butyl ether of the vTyr residue was cleaved for the greater part, probably due to the more time-consuming work-up procedure.

Fortunately, we were able to exchange the residual morpholine for a tertiary amine, *i.e.* *N*-ethyl morpholine (NEM), during purification by chromatography of the crude product on silica (preparative TLC, CH_2Cl_2/MeOH/NEM, 85:15:10) [Scheme 5.2]. Obviously, *N*-ethyl morpholine in **111** will not pose any competition problem during the cyclization reaction.

Scheme 5.2. Deprotection of the termini of linear pentapeptide **110**.



a) $Pd(PPh_3)_4$/morpholine, THF, 45 min; b) preparative TLC on SiO_2, CH_2Cl_2/MeOH/NEM, 73%.

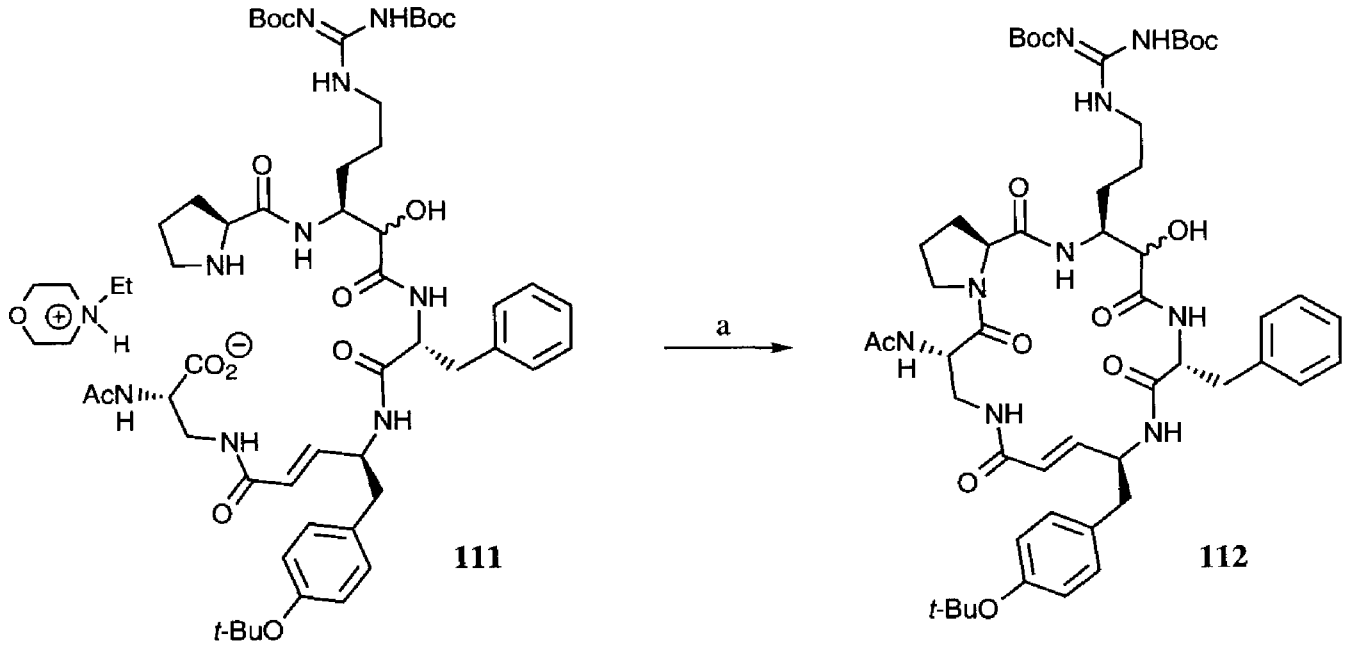
5.2.3. Macrolactamization

In the synthesis of cyclic peptides, the degree of success for macrocyclic coupling depends on the ring size, the type of amino acids residues present, the carboxyl-activating reagent, and the peptide concentration.[5] Cyclodimerization and oligomerization can be prevented by application of high-dilution conditions (1-0.1 mM). In dilute solutions, the rate of the bimolecular reactions is effectively reduced while that of the cyclization, a uni-molecular process, is unaffected. If the macrocyclization is too sluggish, *e.g.* due to the presence of a less reactive *N*-terminal residue like proline or an unfavourable geometry of the linear peptide, racemization of the *C*-terminal amino acid residue can occur. Fortunately, cyclization of pentapeptide **111** is likely to be facilitated by the presence of turn-stabilizing residues such as Pro and D-Phe.

As we had used TBTU successfully for several coupling reactions and as this reagent was also shown to be very effective for couplings involving a proline nitrogen, it was the reagent of choice for the macrolactamization.[6] Although TBTU couplings normally proceed with little or no racemization, addition of HOBt is reported to suppress it completely.[7]

Thus, a 0.5 mM solution of **111** in CH_2Cl_2 was treated with TBTU (3 equiv), HOBt (3 equiv), and DMAP (5 equiv, as base and acylation catalyst), and furnished, after aqueous work-up and preparative TLC, cyclopentapeptide **112** in 61% yield [Scheme 5.3]. Products from side-reactions such as cyclodimerization or epimerization were not detected.

Scheme 5.3. Cyclization of linear pentapeptide **112** with TBTU/HOBt/DMAP.



a) TBTU/HOBt/DMAP, CH_2Cl_2 (0.5 mM), 23 h, 61%.

5.3. Oxidation and Final Deprotection

The penultimate step in the total synthesis of Cyclotheonamide B is oxidation of the α-hydroxy-β-homoarginine unit of **112** to the corresponding α-keto amide derivative. A broad range of oxidizing reagents was studied in collaboration with chemists of N.V. Organon.
Section 5.3.1 gives an overview of the oxidation experiments in model compounds to find optimal reaction conditions. In Section 5.3.2, application of the selected procedure to **112** and final removal of the remaining protecting groups to give Cyclotheonamide B is described.

5.3.1. Oxidation of model compounds

At the time we tested a series of potentially suitable oxidizing reagents, we had just succeeded in the preparation of **51** [Scheme 5.4, Chapter 3.2.2], which was used as model compound.

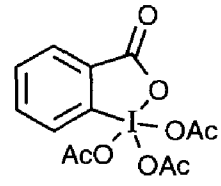
Treatment of **51** with activated dimethyl sulfoxide oxidants (*e.g.* Swern[8] and Pfitzner-Moffatt[9] reagents), often used for the synthesis of α-oxo-β-amino acids, not only affected the hydroxyl group but also the bis-Z guanidine moiety. Besides, the usefulness of the Swern oxidation for peptide-derived substrates is questionable, as epimerization at the centre α to the ketone is reported to occur.[10]
A more recent oxidation method developed by Griffith and Ley in which a catalytic amount of tetra-propylammonium perruthenate (TPAP) is used in combination with a co-oxidant such as *N*-methyl morpholine *N*-oxide, seemed very appealing as the oxidation usually proceeds very fast at room temperature (10-60 min) and the conditions employed are tolerated by a wide variety of functional groups.[11-13] Moreover, the reagent appears to work well for very small substrate quantities when other reagents, such as activated dimethyl sulfoxide oxidants, are particularly difficult to handle. However,

upon treatment of **51** with TPAP, as long as for 3 days, no formation of the keto ester was observed. The starting material was recovered essentially quantitatively. In order to determine whether this failure might be due to the interference of the bis-Z guanidino group, N^{α}-Boc-α-hydroxy-β-homoleucine methyl ester was prepared and subjected to TPAP oxidation. However, also this oxidation attempt failed.

Another oxidation method, recently published, involves an oxammonium salt generated by acid-promoted disproportionation of 2,2,6,6-tetramethyl-piperidine-1-oxide (TEMPO).[14] Unfortunately, application of this method was also without success.

Although oxidation of **51** with Jones' reagent (CrO_3/H_2SO_4 in acetone[15-17]) was the first successful method we encountered (80% crude yield after aqueous work-up), we realized that application of this method in a late stage of our synthesis, employing a substrate that is highly functionalized and provided with acid-labile protecting groups, would not be desirable. Pyridinium dichromate in acetic acid, a more selective and less acidic reagent effected only a low conversion (*ca* 50%).[18]

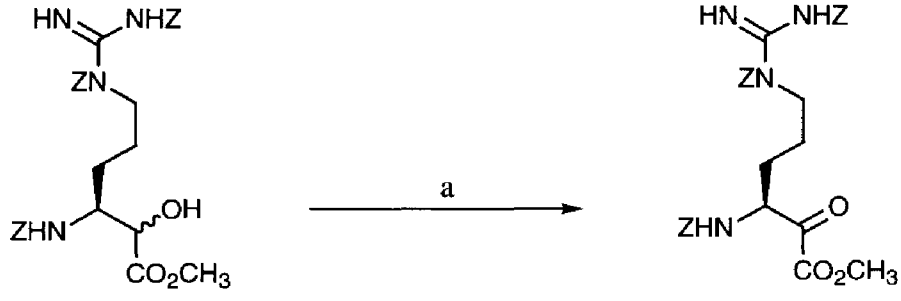
Since the initial report of Dess and Martin about the preparation and use of their periodinane oxidizing agent, *i.e.* 1,1,1-triacetoxy-1,1-dihydro-1,2-benziodoxol-3(1*H*)-one (**114**) a large number of chemists have found the "Dess-Martin oxidation" to be one of the most selective and mild methods available, very suitable for the oxidation of primary alcohols to aldehydes and secondary alcohols to ketones in highly functionalized and sensitive substrates.[19-22]



114

Indeed, reaction of **51** with periodinane **114** yielded, after work-up, the desired keto ester **113** in good yield [Scheme 5.4]. However, attempts to purify **113** on silica were unsuccessful, probably due to the sensiti ity of the α-keto ester. The arginine containing peptide **66** with a mono-Z protected guanidino group was also successfully oxidized in good yield [Scheme 5.5, see also Chapter 3.2.3].

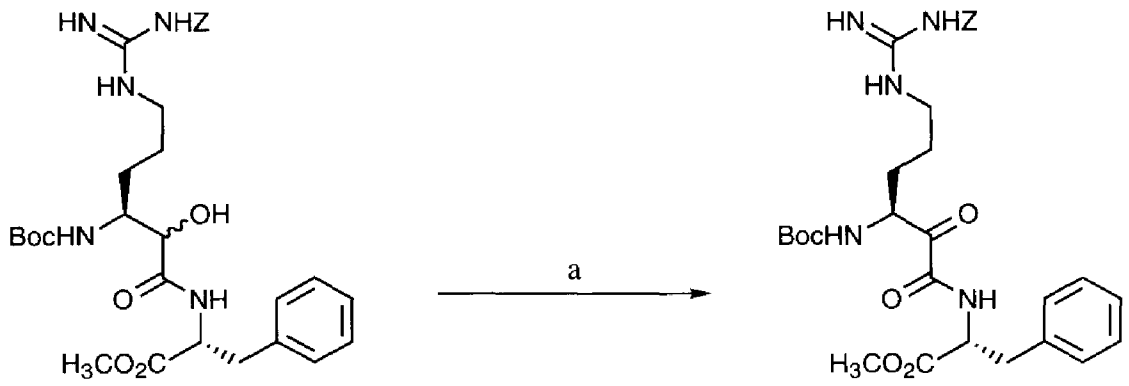
Scheme 5.4. Oxidation of a bis-Z protected α-hydroxy-β-homoarginine derivative.



51 **113**

a) **114**, CH_2Cl_2, 1 h, 81% (crude yield).

Scheme 5.5. Oxidation of a mono-Z protected α-hydroxy-β-homoarginine derivative.

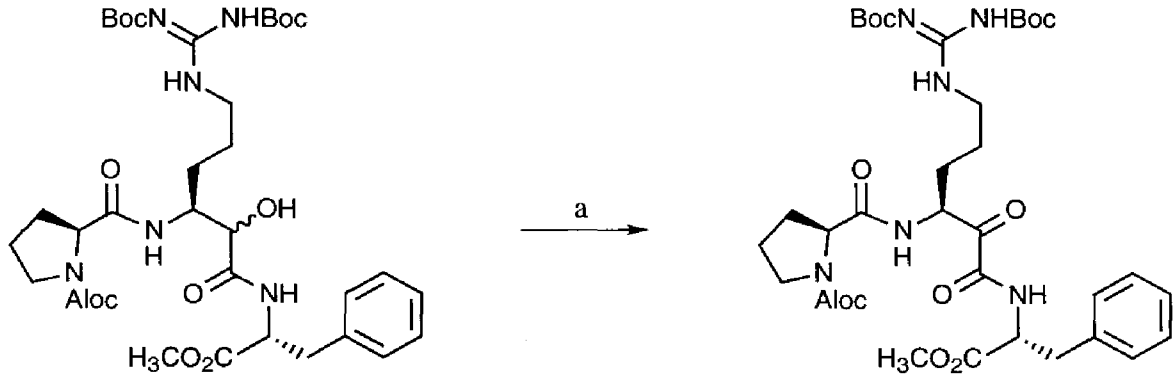


66 **67**

a) **114**, CH_2Cl_2, 1.5 h, 73% (crude yield).

Since we had abandoned the guanidine Z-protection it became necessary to verify if the Dess-Martin oxidation was also compatible with the new bis-Boc protecting group strategy. As it has been reported that rate of the oxidation reaction is markedly increased by the addition of *t*-butanol[20] tripeptide **96** was treated with **114**/*t*-butanol to furnish, after 45 min reaction time, aqueous work-up and chromatography on silica, pure α-keto amide **115** in 78% yield [Scheme 5.6].

Scheme 5.6. Oxidation of a bis-Boc protected α-hydroxy-β-homoarginine derivative.



96 **115**

a) **114**/*t*-BuOH, CH_2Cl_2, 45 min, 78%.

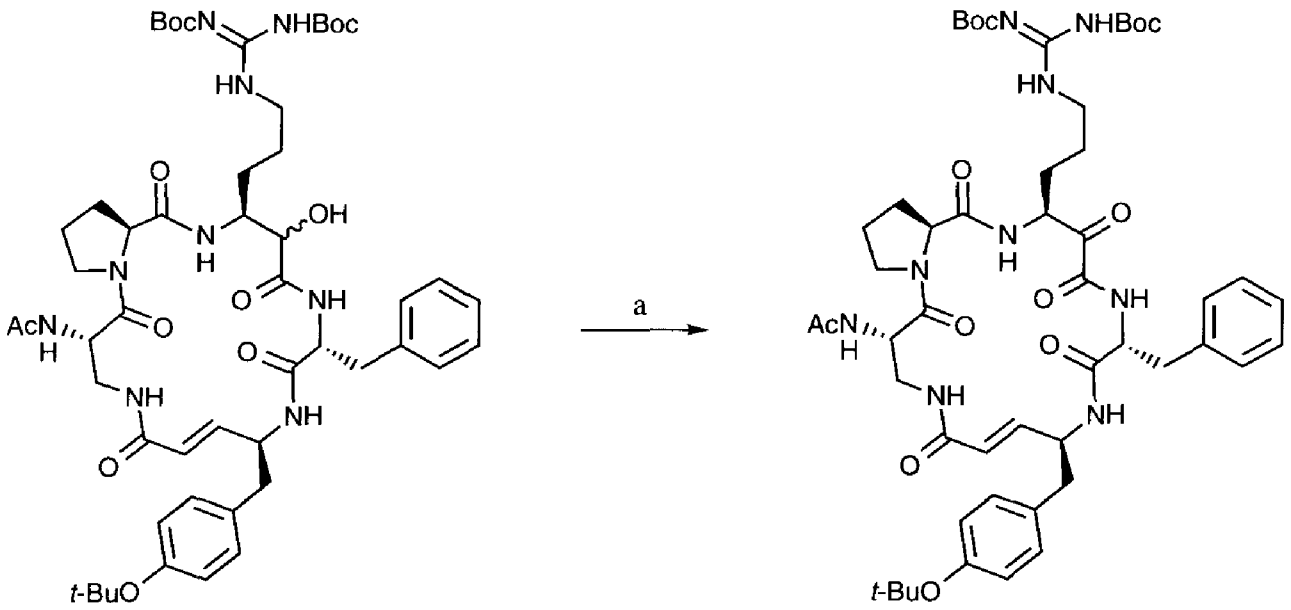
Thus, the Dess-Martin oxidation appeared to be the method of choice to convert α-hydroxy amide **112** into the corresponding α-hydroxy amide.

5.3.2. Oxidation and deprotection of cyclopentapeptide 112

Exposure of **112** to the optimized oxidation conditions (**114**/*t*-butanol) did not furnish the oxidized product. Even after treatment for 24 hours at 40 °C with a large excess of **114**/*t*-butanol (2.5 equiv) in CH_2Cl_2/MeCN starting material (≤10%, as shown by ^{1}H-NMR) was still present [Scheme 5.7].

Similar observations have been described by Maryanoff[23] and Wipf[24] for the oxidation of the α-hydroxy-β-homoarginine residue in their cyclopentapeptide. Presumably, the ring geometry of the 19-membered macrocycle forces the hydroxyl group into a sterically and/or electronically unfavourable position, as can be concluded from the observation that linear α-hydroxy-β-homoarginine derivatives are oxidized much faster (*vide supra*). Noteworthy in this context is also the observation that an 18-membered ring analogue of Cyclotheonamide was rapidly oxidized, whereas three analogues having a 19-membered ring were oxidized much slower [Chapter Six].

Scheme 5.7. Oxidation of cyclopentapeptide **112**.



112 **116**

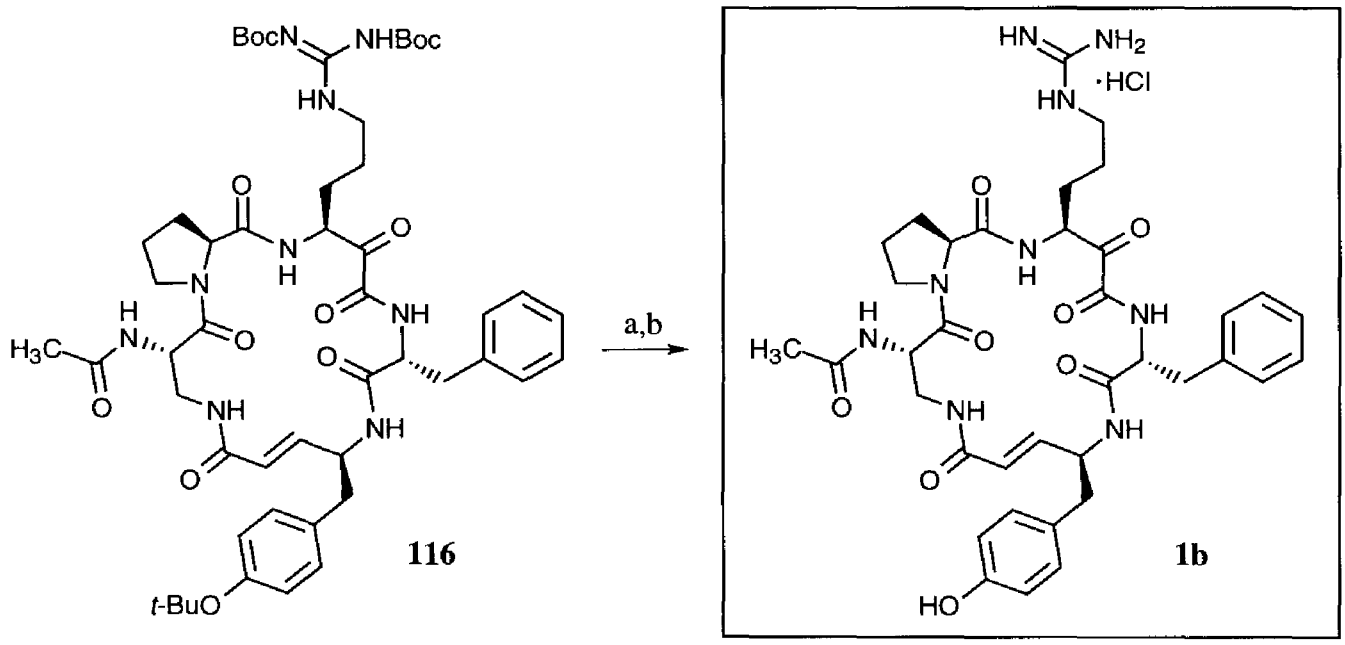
a) Dess-Martin periodinane/*t*-BuOH, CH_2Cl_2/MeCN, 40 °C, 24 h, *ca* 90% (based on ^{1}H-NMR).

The crude oxidation product **116** was treated with TFA/thioanisole[25] at room temperature during 105 min to deprotect the arginine and tyrosine units. The resulting material was purified by reverse-phase HPLC, to give Cyclotheonamide B in 51% yield from **112** [Scheme 5.8]. Synthetic Cyclotheonamide B was fully characterized (^{1}H-NMR, ^{13}C-NMR, HH-cosy, CH-cosy, FAB-MS, optical rotation, and bioassay) and was identical in all respects to the natural product.

Comparative 400 MHz ^{1}H-NMR spectra of natural Cyclotheonamide A (kindly provided by professor Fusetani) and synthetic Cyclotheonamide B are depicted in Figure 5.1. The differences observed are solely due to the presence of a N^{α}-formyl group at the diaminopropanoic acid residue in Cyclotheonamide A and a N^{α}-acetyl group in Cyclotheonamide B.

Noteworthy is the ^{13}C-chemical shift value of the α-carbon atom of the β-homoarginine unit ($\delta_{C\alpha}$= 97.2 ppm) caused by the presence of the hydrated form (*gem*-diol) rather than the α-keto amide form. Formation of adducts of Cyclotheonamide and protic solvents such as water, methanol and ethanol is also observed by FAB-MS. Formation of these adducts might contribute to the stability towards epimerization of the racemization-prone α-oxo-β-homoarginine residue, and is the mechanistic rationale for the high affinity of the natural product for serine proteases.

Scheme 5.8. Deprotection of cyclopentapeptide **116** to give Cyclotheonamide B (**1b**).



a) TFA/thioanisole, 105 min; b) HPLC: MeCN/H_2O/phosphate buffer; desalting: MeCN/H_2O/HCl, 51% from **112**.

Figure 5.1. Comparative 400 MHz ^{1}H-NMR spectra of natural Cyclotheonamide A (top panel) and synthetic Cyclotheonamide B (bottom panel) in D_2O.

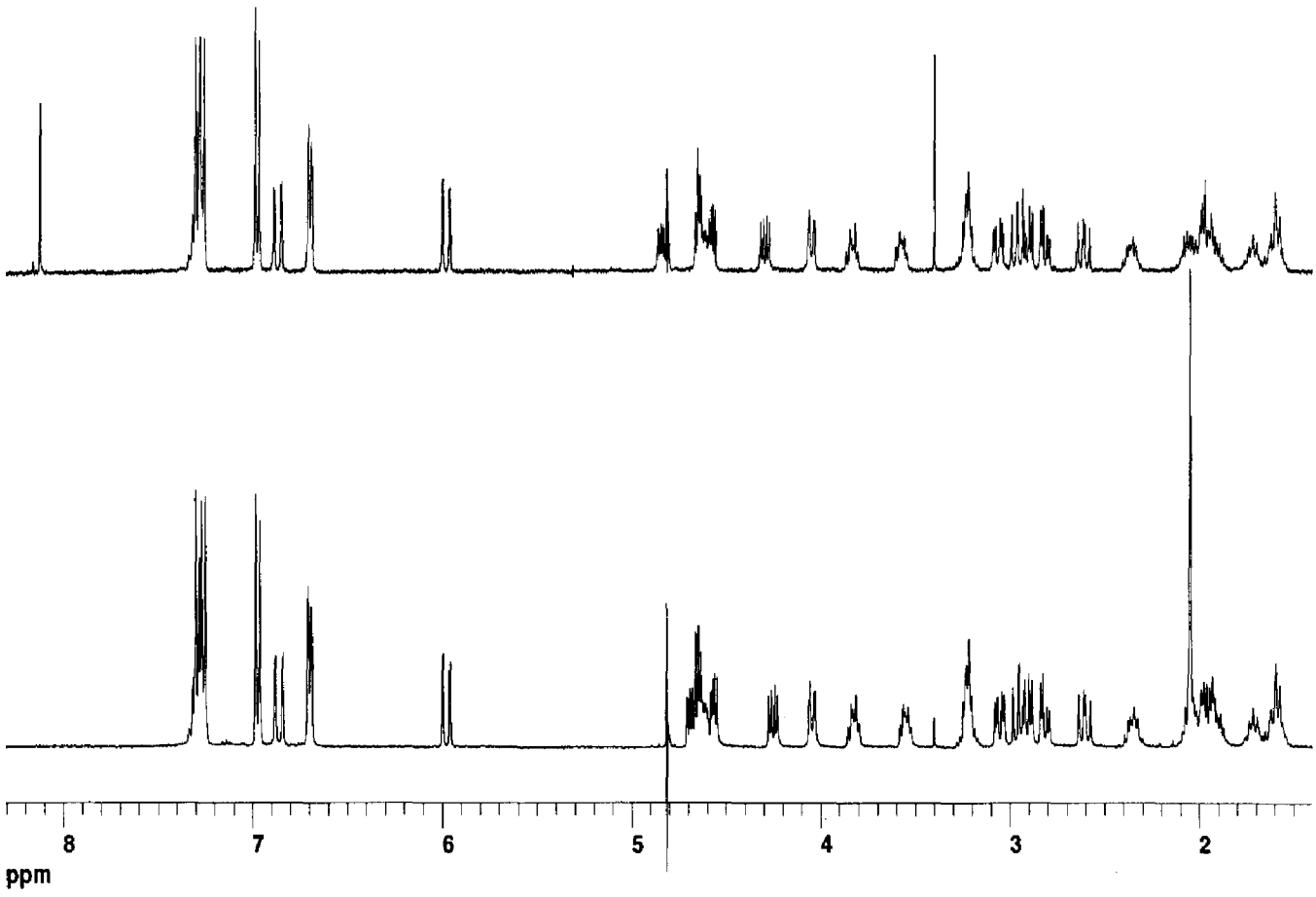
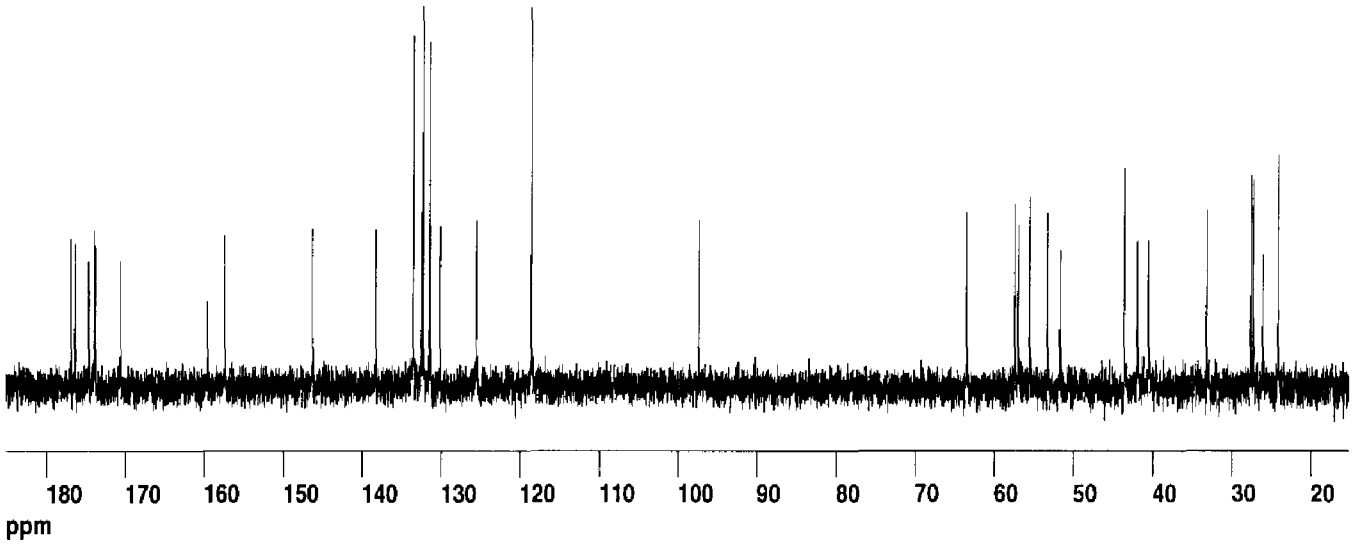


Figure 5.2. 100 MHz ^{13}C-NMR spectrum of synthetic Cyclotheonamide B in D_2O.



5.4. Conclusions

Elaboration of the two key intermediates Segment A and Segment B, in a five step sequence, into Cyclotheonamide B proceeded smoothly and gave the target compound in 19% yield from Segment A. Synthetic Cyclotheonamide B was found to be identical in all respects to the natural product.
A concise summary of our overall route to Cyclotheonamide B, together with a comparison with the synthetic efforts of other research groups will be given in Chapter Eight.

5.5. Experimental

Detailed general experimental information is given in Section 3.6.

Allyl 2(*S*)-acetylamino-3-[[4(*S*)-[[2(*R*)-[[2(*R,S*)-hydroxy-3(*S*)-[[[1-(allyloxycarbonyl)pyrrolidin-2(*S*)-yl]carbonyl]amino]-6-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]-methyl]amino]hexanoyl]amino]-3-phenylpropanoyl]amino]-5-[4-(*tert*-butyloxy)phenyl]pent-2-(*E*)-enoyl]amino]propanoate (110)

To a stirred solution of tripeptide **97** (267 mg, 0.365 mmol) and dipeptide **109** (206 mg, 0.400 mmol + lutidine) in CH_2Cl_2 (10 mL), was added DiPEA (140 µL, 0.804 mmol) followed by TBTU (129 mg, 0.402 mmol). After 3 h the reaction mixture was diluted with EtOAc (100 mL), and sequentially washed with, H_2O (3x), aqueous $NaHCO_3$ (5%, 3x), H_2O, aqueous $KHSO_4$ (6%, 3x), H_2O (2x) and brine, dried (Na_2SO_4), filtrated, and concentrated *in vacuo* to give, after preparative TLC (EtOAc/ CH_2Cl_2, 6:4), **110** as a white foam (356 mg, 85.3%).

^{1}H-NMR (DMSO-d_6, 400.1 MHz): 1.07-1.43 (m, 4H, hArg γ- and δ-H), 1.19 (s, 9H, *t*-Bu), 1.39 (s, 9H, Boc), 1.47 (s, 9H, Boc), 1.67-1.80 (m, 3H, Pro β- and γ-H), 1.81 (s, 3H, Ac), 1.97 (m, 1H, Pro β-H), 2.53-2.72 (m, 3H, Phe β-H, vTyr δ-H), 2.82 (m, 1H, Phe β-H), 3.19 (m, 2H, hArg ε-H), 3.27-3.40 (m, 3H, Pro δ-H, Dpr β-H), 3.51 (m, 1H, Dpr β-H), 3.82 (m, 1H, hArg α-H), 3.90 (m, 1H, hArg β-H), 4.18 (m, 1H, Pro α-H), 4.31-4.60 (m, 7H, Phe α-H, vTyr γ-H, Dpr α-H and 2x $OC\underline{H}_2CH=CH_2$), 5.03-5.32 (m, 4H, 2x $OCH_2CH=C\underline{H}_2$), 5.73-5.92 (m, 4H, hArg OH, vTyr α-H and 2x $OCH_2C\underline{H}=CH_2$), 6.60 (dd, *J*=15.2 and 5.3, 1H, vTyr β-H), 6.87 (part of AB-system, *J*=8.4, vTyr aryl), 6.89-6.97 (m, 2H, Phe aryl), 7.08-7.24 (m, 5H, Phe aryl, vTyr aryl), 7.47-7.59 (m, 2H, hArg β-NH, Phe α-NH), 8.14-8.29 (m, 4H, hArg ε-NH, vTyr γ-NH, Dpr α- and β-NH) and 11.50 (bs, 1H, hArg ω-NH). ^{13}C-NMR ($CDCl_3$): 22.7 ($C(O)\underline{C}H_3$), 24.5 (δ-hArg), 25.6 (γ-hArg), 28.0 ($OC(\underline{C}H_3)_3$), 28.2 ($C(O)OC(\underline{C}H_3)_3$), 28.7 ($C(O)OC(\underline{C}H_3)_3$), 29.5 (γ-Pro), 31.1 (β-Pro, b), 38.4 (β-Phe), 39.4 (ε-hArg, b), 40.6 and 40.8 (δ-vTyr and β-Dpr), 46.9 (δ-Pro, b), 51.1 (α-Phe), 51.6 (γ-vTyr), 53.2 (β-hArg), 54.8 (α-Dpr), 60.8 (α-Pro), 66.2 and 66.4 ($O\underline{C}H_2CH=CH_2$, Aloc and ester), 71.8 (α-hArg), 78.2 ($O\underline{C}(CH_3)_3$), 79.2 ($C(O)O\underline{C}(CH_3)_3$), 83.0 ($C(O)O\underline{C}(CH_3)_3$), 117.7 ($OCH_2CH=\underline{C}H_2$, Aloc), 118.7 ($OCH_2CH=\underline{C}H_2$, ester), 122.4 (α-vTyr), 123.9 (aryl-3 vTyr), 127.0 (aryl-4 Phe), 128.5 (aryl-2 Phe), 129.3 (aryl-3 Phe), 129.8 (aryl-2 vTyr), 131.2 (aryl-1 vTyr), 131.5 and 132.5 ($OCH_2\underline{C}H=CH_2$, Aloc and ester), 136.5 (aryl-1 Phe), 143.2 (β-vTyr), 153.1 (C(O)O, Boc), 154.0 (aryl-4 vTyr), 154.8 and 155.4 (C(O)O, Aloc rotam), 156.1 (C=N), 163.3 (C(O)O, Boc), 167.7 (C(O)NH, Phe-Dpr), 170.4 (C(O)NH), 170.4 ($C(O)CH_3$), 171.2, 172.4 and 172.6 ($\underline{C}$(O)OAll and 2x C(O)NH).

***N*-Ethyl morpholinium 2(*S*)-acetylamino-3-[[4(*S*)-[[2(*R*)-[[2-(*R*,*S*)-hydroxy-3(*S*)-[[[1-(allyloxycarbonyl)pyrrolidin-2(*S*)-yl]carbonyl]amino]-6-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]methyl]amino]hexanoyl]amino]-3-phenylpropanoyl]amino]-5-[4-(*tert*-butyloxy)-phenyl]pent-2(*E*)-eno-yl]amino]propanoate (111)**

To a stirred solution of **110** (316 mg, 0.270 mmol) and morpholine (1.18 mL, 13.5 mmol) in THF (38 mL) was added $Pd(PPh_3)_4$ (20.1 mg, 0.017 mmol). After 45 min the volatiles were evaporated *in vacuo*. The crude product was purified by preparative TLC (CH_2Cl_2/MeOH/NEM, 85:15:10), to give **111** as an off-white powder (224 mg; 72.9%).

^{1}H-NMR (DMSO-d_6): 0.99 (t, *J*=6.7, 3H, *N*-$CH_2C\underline{H}_3$ NEM), 1.05-2.00 (m, 8H, Pro β- and γ-H, hArg γ- and δ-H), 1.20 (s, 9H, *t*-Bu), 1.38 (s, 9H, Boc), 1.48 (s, 9H, Boc), 2.23-2.89 (m, 6H, *N*-$C\underline{H}_2CH_3$ NEM), 1.81 (s, 3H, Ac), 2.55-3.05 (m, 4H, Phe β-H, vTyr δ-H), 3.07-3.61 (m, 10H, Pro δ-H, hArg ε-H, Dpr β-H, *O*-CH_2 NEM), 3.70 (m, 1H, Pro α-H), 3.81-4.00 (m, 2H, hArg α- and β-H), 4.08 (m, 1H, Dpr α-H), 4.41-4.63 (m, 2H, Phe α-H, vTyr γ-H), 5.92 (bd *J*=15.3, 1H, vTyr α-H), 6.02 (bs, 1H, hArg OH), 6.59 (dd, *J*=15.3 and 4.7, 1H, vTyr β-H), 6.87 (part of AB-system, *J*=8.2, 2H, vTyr aryl), 6.90-7.00 (m, 2H, Phe aryl), 7.03-7.28 (m, 5H, Phe aryl, vTyr aryl), 7.60 (d, *J*=8.2, 1H, NH), 7.79 (d, *J*=7.0, 1H, NH), 7.96 (bt, *J*=6.0, 1H, Dpr β-NH), 8.0.8 (d, *J*=8., 1H, NH), 8.18-8.45 (bt, 2H, hArg ε-NH, NH) and 11.49 (bs, 1H, hArg ω-NH). FAB-HRMS: calcd for $[C_{51}H_{75}N_9O_{13} + H]^+$ 1022.5563, found 1022.5546.

***(2S,5S,11S,14R,17(R,S),18S)-N*-[11-[4-(*tert*-Butyloxy)benzyl]-14-benzyl-17-hydroxy-18-[[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]methyl]amino]propyl]-4,8,13,16,20-pentaoxo-2,3,4,5,6,7,8,11,12,13,14,15,16,17,18,19,20,20a-octadecahydro-1*H*-3a,7,12,15,19-pentaaza-cyclopentacyclononadecen-5-yl]acetamide (112)**

To a stirred solution of **111** (223 mg, 0.196 mmol) in CH_2Cl_2 (393 mL) was added DMAP (96.5 mg,

0.790 mmol), HOBt (53.0 mg, 0.393 mmol) and TBTU (126.6 mg, 0.393 mmol). After 1.5 h again DMAP (24.1 mg, 0.197 mmol), HOBt (26.5 mg, 0.197 mmol) and TBTU (63.1 mg, 0.197 mmol) were added. After 23 h the solvent was slowly evaporated *in vacuo* at ambient temperature and the residue was partitioned between EtOAc and H_2O. The organic layer was sequentially washed with H_2O (2x), aqueous $NaHCO_3$ (5%, 2x), H_2O (2x), aqueous $KHSO_4$ (6%, 2x), H_2O (2x) and brine, dried (Na_2SO_4), filtrated, and concentrated *in vacuo* to give, after preparative TLC (CH_2Cl_2/THF/EtOH, 70:30:1.5), **112** as a white foam (119 mg, 60.6%). The ^{1}H-NMR spectrum of **112** in $CDCl_3$ was poorly resolved.

^{1}H-NMR ($CDCl_3$): 1.28 (s, 9H, *t*-Bu), 1.35-2.00 (m, 7H, Pro γ-H and δ-H, hArg γ- and δ-H), 1.45 (s, 9H, Boc), 1.51 (s, 9H, Boc), 1.92 (s, 3H, Ac), 2.12 (m, 1H, Pro β-H), 2.51 (m, 1H, vTyr δ-H), 2.55 (m, 4H, vTyr δ-H, Phe β-H, Dpr β-H), 3.21 (m, 1H, hArg ε-H), 3.42 (m, 1H, Pro δ-H), 3.51-3.79 (m, 3H, hArg ε-H and OH, Pro δ-H), 3.99 (m, 1H, hArg α-H), 4.15-4.36 (m, 3H, hArg β-H, Dpr β-H, Phe α-H), 4.40 (m, 3H, Pro α-H), 4.53-4.88 (m, 2H, vTyr γ-H, Dpr α-H), 5.10 (bd, *J*=8.2, 1H, vTyr γ-NH), 5.88 (dd, *J*=15.7 and 2.3, 1H, vTyr α-H), 6.58 (bd, *J*=8.0, 1H, Dpr α-NH), 6.63 and 6.71 (AB-system, *J*=8.2, 4H, vTyr aryl), 6.73 (dd overlapping, 1H, vTyr β-H), 7.00-7.18 (m, 3H, Phe aryl and α-NH), 7.19-7.49 (m, 3H, Phe aryl), 7.78 (bd, *J*=9.3, 1H, hArg β-NH), 7.92 (bd, *J*=9.5, 1H, Dpr β-NH), 8.42 (bt, *J*=6.0, 1H, hArg ε-NH) and 11.54 (bs, 1H, hArg ω-NH).

^{1}H-NMR (DMSO-d_6, 400.1 MHz): 1.22 (s, 9H, *t*-Bu), 1.24 (m, 1H, hArg γ-H), 1.39 (s, 9H, Boc), 1.46 (s, 9H, Boc), 1.39-1.58 (m, 3H, hArg γ- and δ-H), 1.81 (s, 3H, Ac), 1.71-1.88 (m, 2H, Pro γ-H), 1.92 (m, 1H, Pro β-H), 2.09 (m, 1H, Pro β-H), 2.39-2.61 (m, 3H, Phe β-H, vTyr δ-H), 2.69 (m, 1H, Dpr β-H), 2.97 (m, 1H, vTyr δ-H), 3.28 (m, 2H, hArg ε-H), 3.39 (m, 1H, Pro δ-H), 3.59 (m, 1H, Pro δ-H), 3.92-4.09 (m, 4H, hArg α-, δ-H and OH, Dpr β-H), 4.41-4.55 (m, 3H, Pro α-H, vTyr γ-H, Dpr α-H), 4.60 (m, 1H, Phe α-H), 5.95 (dd *J*=15.4 and 2.2, 1H, vTyr α-H), 6.68 (dd, *J*=15.3 and 2.0, 1H, vTyr β-H), 6.75-6.83 (m, 2H, Phe aryl), 6.92 and 7.29 (AB-system, *J*=8.5, 4H, vTyr aryl), 7.05-7.12 (m, 3H, Phe aryl), 7.90 (d, *J*=9.7, 1H, Dpr α-NH), 8.08 (d, *J*=8.3, 1H, Phe α-NH), 8.19 (d, *J*=6.7, 1H, Dpr β-NH), 8.27 (d, *J*=8.6, 1H, vTyr γ-NH), 8.32 (bt, *J*=6.0, 1H, hArg ε-NH), 8.45 (d, *J*=10.6, 1H, hArg β-NH) and 11.53 (bs, 1H, hArg ω-NH).

^{13}C-NMR ($CDCl_3$): 22.7 (C(O)C$\underline{H}_3$), 24.5 (δ-hArg), 26.6 (γ-hArg), 27.9 (OC($\underline{C}H_3$)$_3$), 28.3 (C(O)O-C($\underline{C}H_3$)$_3$), 28.6 (C(O)OC($\underline{C}H_3$)$_3$), 27.0 (γ-Pro), 30.6 (β-Pro), 38.3 (β-Phe), 39.8 (β-Dpr), 40.2 (δ-vTyr), 40.4 (ε-hArg), 47.9 (δ-Pro), 49.2 (α-Phe), 50.1 (γ-vTyr), 53.0 (β-hArg), 55.7 (α-Dpr), 60.3 (α-Pro), 72.3 (α-hArg), 78.3 (O$\underline{C}$(CH$_3$)$_3$), 79.4 (C(O)O$\underline{C}$(CH$_3$)$_3$), 82.9 (C(O)O$\underline{C}$(CH$_3$)$_3$), 123.8 (aryl-3 vTyr), 124.2 (α-vTyr), 127.1 (aryl-4 Phe), 128.5 (aryl-2 Phe), 129.3 (aryl-3 Phe), 129.7 (aryl-2 vTyr), 129.9 (aryl-1 vTyr), 136.1 (aryl-1 Phe), 141.2 (β-vTyr), 153.0 (C(O)O, Boc), 154.3 (aryl-4 vTyr), 156.7 (C=N), 163.2 (C(O)O, Boc), 165.0 (C(O)NH, vTyr-Dpr), 169.1 (C(O)NH), 169.9 (C(O)CH$_3$), 170.1 (C(O)NH), 171.1 (C(O)NH) and 172.6 (C(O)NH). FAB-HRMS: calcd for $[C_{51}H_{73}N_9O_{12} + H]^+$ 1004.5457, found 1004.5452.

***(2S,5S,11S,14R,18S)-N*-[11-[4-(*tert*-Butyloxy)benzyl]-14-benzyl-18-[[[(*tert*-butyloxycarbonyl imino[(*tert*-butyloxycarbonyl)amino]methyl]amino]propyl]-4,8,13,16,17,20-hexaoxo-2,3,4,5,6,7,8,11,12,13,14,15,16,17,18,19,20,20a-octadecahydro-1*H*-3a,7,12,15,19-pentaaza-cyclopentacyclononadecen-5-yl]acetamide (116)**

A solution of **112** (155 mg, 0.154 mmol), Dess-Martin periodinane (**114**) (177 mg, 0.417 mmol) and *t*-BuOH (37 μL, 0.386 mmol) in CH_2Cl_2/MeCN (7.75 mL, 2:1) was stirred at 40 °C for 23 h. The

reaction mixture was allowed to cool to room temperature, was diluted with EtOAc (20 mL) and vigorously shaken with a solution of $NaS_2O_3 \cdot 5H_2O$ (4.0 g) in saturated aqueous $NaHCO_3$ (10 mL) until a clear bi-phasic system appeared. The organic layer was sequentially washed with saturated aqueous $NaHCO_3$, H_2O and brine, dried (Na_2SO_4), filtrated, and concentrated *in vacuo* to give **116** as a white foam (157 mg, *ca* 90%). The ^{1}H-NMR spectrum showed the presence of a small amount (≤10%) of starting material.

^{1}H-NMR ($CDCl_3$): 1.32 (s, 9H, *t*-Bu), 1.35-2.27 (m, 8H, Pro γ-H and δ-H, hArg γ- and δ-H), 1.47 (s, 9H, Boc), 1.49 (s, 9H, Boc), 1.97 (s, 3H, Ac), 2.50-2.81 (m, 2H, vTyr δ-H), 2.88-3.67 (m, 7H, Pro δ-H, hArg ε-H, Phe β-H, Dpr β-H), 4.13-4.40 (m, 3H, hArg β-H, Phe α-H, Dpr δ-H), 4.46 (m, 3H, Pro α-H), 4.67 (m, 1H, vTyr γ-H), 4.87 (m, 1H, Dpr α-H), 5.47 (bd, *J*=8.2, 1H, vTyr γ-NH), 5.62 (dd, *J*=15.3 and 1.8, 1H, vTyr α-H), 6.77-6.92 (m, 5H, vTyr aryl, Dpr α-NH), 6.92 (dd overlapping, 1H, vTyr β-H), 7.1-7.23 (m, 2H, Phe aryl), 7.23-7.39 (m, 3H, Phe aryl), 7.45 (bd, *J*=8.7, 1H, NH), 7.55-7.82 (m, 2H, 2x NH), 8.42 (bt, *J*=6.0, 1H, hArg ε-NH) and 11.53 (bs, 1H, hArg ω-NH). FAB-HRMS: calcd for $[C_{51}H_{71}N_9O_{12} + H]^+$ 1002.5300, found 1002.5265.

***(2S,5S,11S,14R,18S)-N*-[11-(4-Hydroxybenzyl)-14-benzyl-18-[[imino(amino)methyl]amino]-propyl)]-4,8,13,16,17,20-hexaoxo-2,3,4,5,6,7,8,11,12,13,14,15,16,17,18,19,20,20a-octadeca-hydro-1*H*-3a,7,12,15,19-pentaaza-cyclopentacyclononadecen-5-yl]acetamide (1b)**

To a stirred suspension of **116** (69.1 mg, *ca* 67.8 μmol, *vide supra*) in thioanisole (0.3 mL) was added TFA (2.7 mL). The resulting solution was stirred for 105 min. Subsequently, the volatiles were evaporated *in vacuo*. The crude product was purified by reverse phase HPLC; column: Supelco LC-18 DB 250x50 mm; mobile phase: (MeCN/H_2O, 3:2)/H_2O/phosphate buffer (0.5 M, pH= 2.1), gradient from 20:60:20 to 60:20:20 in 42 min; flow: 20 mL/min. The product was desalted on the same column (MeCN/H_2O, 3:2)/H_2O/HCl_{aq} (0.1 N), 0:80:20 15 min, then 80:18:2), and lyophilized to give Cyclotheonamide B·1.1HCl·9.6H_2O (based on peptide content) as a fluffy white powder (32.8 mg, 50.8% from **116**); analytical HPLC, ≥96.5%; $[\alpha]_D^{23}$ −13.7° (*c*=0.2, MeOH), lit $[\alpha]_D^{23}$ −13.6° (*c*=0.2, MeOH).[26]

^{1}H-NMR (D_2O, 400.1 MHz): 1.47-1.60 (m, 2H, kArg γ- and δ-H), 1.67 (m, 1H, kArg δ-H), 1.80-2.09 (m, 4H, Pro β- and γ-H, kArg γ-H), 1.99 (s, 3H, Ac), 2.30 (m, 1H, Pro β-H), 2.54 (dd, *J*=14.0 and 10.3, 1H, vTyr δ-H), 2.77 (dd, *J*=13.7 and 4.6, 1H, Phe β-H), 2.86 (dd, *J*=13.7 and 6.0, 1H, Phe β-H), 2.89 (t, *J*=11.4, 1H, Dpr β-H), 2.99 (dd, *J*=14.0 and 4,9, 1H, vTyr δ-H), 3.16 (m, 2H, kArg ε-H), 3.49 (m, 1H, Pro δ-H), 3.76 (m, 1H, Pro δ-H), 3.98 (dd, *J*=10.9 and 2.4, 1H, kArg β-H), 4.20 (dd, *J*=12.8 and 5.9, 1H, Dpr β-H), 4.50 (m, 1H, Pro α-H), 4.54 (m, 1H, vTyr γ-H), 4.58 (m, 1H, Phe α-H), 4.62 (m, 1H, Dpr α-H), 5.91 (dd, *J*=15.6 and 2.1, 1H, vTyr α-H), 6.63 (m, 2H, Phe aryl), 6.80 (dd, *J*=15.6 and 2.6, 1H, vTyr β-H), 6.91 (part of AB-system, *J*=8.4, 2H, vTyr aryl), 7.17-7.29 (m, 5H, Phe aryl, vTyr aryl), ^{13}C-NMR ($CDCl_3$): 24.0 (C(O)$\underline{C}H_3$), 25.9 (γ-kArg), 27.2 (δ-kArg), 27.5 (γ-Pro), 33.0 (β-Pro), 40.4 (δ-vTyr), 41.7 (β-Dpr), 41.8 (β-Phe), 43.4 (ε-hArg), 51.4 (δ-Pro), 53.1 (α-Dpr), 55.4 (γ-vTyr), 56.8 (α-Phe), 57.2 (β-kArg), 63.3 (α-Pro), 97.2 (α-kArg), 118. 4 (aryl-3 vTyr), 125.3 (α-vTyr), 130.0 (aryl-4 Phe), 131.2 (aryl-3 Phe), 132.1 (aryl-2 Phe), 132.4 (aryl-1 vTyr), 133.3 (aryl-2 Phe), 138.0 (aryl-1 Phe), 146.1 (β-vTyr), 157.2 (aryl-4 vTyr), 159.4 (C=N), 170.3 (C(O)NH), 173.6 (C(O)NH), 173.8 (C(O)NH), 174.4 (C(O)NH), 176.1 (C(O)NH) and 176.7 (C(O)NH). FAB-MS *m/z* 746 (MH^+) and 764 ($MH^+ + H_2O$).

Oxidation experiments with model compounds

Methyl 2(*R*)-[[2-oxo-3(*S*)-[(*tert*-butyloxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)amino]methyl]amino]hexanoyl]amino]-3-phenylpropanoate (67)

To a stirred solution of **66** (90 mg, 0.15 mmol) and HOAc (10 μL, 0.17 mmol) in CH_2Cl_2 (1 mL) was added a solution of Dess-Martin periodinane **114** (0.22 g, 0.52 mmol) in CH_2Cl_2 (2.0 mL). After 90 min, Et_2O (60 mL) and a solution of $NaHCO_3$ (0.25 g) and $Na_2SO_3 \cdot 5H_2O$ (0.75 g) in H_2O (10 mL) were added. The mixture was shaken vigorously to give a clear bi-phasic system. The layers were separated, the aqueous layer was extracted with Et_2O (40 mL), and the combined etheral solutions were washed with aqueous $NaHCO_3$ (5%) and brine, dried (Na_2SO_4), filtrated, and concentrated *in vacuo* to give **67** as a white foam (65 mg; 72.5%).

^{1}H-NMR ($CDCl_3$): 1.40 and 1.42 (2x s, 9H, Boc), 1.29-1.87 (m, 4H, kArg γ- and δ-H), 2.95-3.27 (m, 4H, kArg ε-H, Phe β-H), 3.73 (m, 3H, OCH_3), 4.85 (m, 1H, Phe α-H), 4.9-5.15 (m, 3H, kArg γ- and β-H, $OC\underline{H}_2Ph$), 5.46 (m, 1H, kArg β-NH) and 6.96-7.45 (m, 13H, aryl, Phe α-NH, guanidine NH).

Methyl 2-oxo-3(*S*)-[(phenylmethoxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)-amino]methyl](phenylmethoxycarbonyl)amino]hexanoate (113)

To a stirred solution of α-hydroxy ester **51** (150 mg, 0.25 mmol) in CH_2Cl_2 (5 mL) was added Dess-Martin periodinane **114** (250 mg, 0.5 mmol). After 2 h the reaction mixture was diluted with diethyl ether (50 mL) followed by a solution of $NaHCO_3$ (0.9 g) and $Na_2S_2O_3 \cdot 5H_20$ (2.9 g) in H_2O (40 mL). The bi-phasic system was vigorously shaken until both layers became clear. The separated organic layer was washed with aqueous $NaHCO_3$ (25 mL) and brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo* to give **113** as an off-white foam (125 mg; 80.7%).

^{1}H-NMR ($CDCl_3$): 1.50-2.03 (m, 4H, γ- and δ-H), 3.77 (s, 3H, OCH_3), 3.77-4.10 (m, 2H, ε-H), 4.79-4.97 (m, 1H, α-H), 5.07 (s, 2H, $OC\underline{H}_2Ph$), 5.11 (s, 2H, $OC\underline{H}_2Ph$), 5.20 (s, 2H, $OC\underline{H}_2Ph$), 7.4 (d, *J*=7.8, β-NH), 7.18-7.47 (m, 15H, aryl), 9.25 (bs, 1H, ω-NH) and 9.44 (bs, 1H, ω'-NH). ^{13}C-NMR ($CDCl_3$): 24.6 (δ-C), 26.7 (γ-C), 43.8 (ε-C), 52.9 ($O\underline{C}H_3$), 56.9 (β-C), 66.9 ($O\underline{C}H_2Ph$, 2x), 68.8 ($O\underline{C}H_2Ph$), 127.6 (aryl-4), 128.2 (aryl-3,5), 128.7 (aryl-2,6), 134.4 (aryl-1), 136.6 (aryl-1), 136.8 (aryl-1), 155.2 (C(O)O, Z, 155.6 (C(O)O, Z), 160.3 (C=N), 160.9 ($\underline{C}(O)OCH_3$), 163.4 (C(O)O, Z) and 199.8 (α-C).

Methyl 2(*R*)-[[2-oxo-3(*S*)-[[[1-(allyloxycarbonyl)pyrrolidin-2(*S*)-yl]carbonyl]amino]-6-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]methyl]amino]hexanoyl]amino]-3-phenylpropanoate (115)

To stirred solution of **96** (82.1 mg, 0.110 mmol) in CH_2Cl_2 (2.1 mL) was added *t*-BuOH (13.5 μL, 0.143 mmol) and Dess-Martin periodinane **114** (69.9 mg, 0.765 mmol). After 1.5 h the reaction mixture was poured onto a mixture of EtOAc (8 mL) and $Na_2S_2O_3 \cdot 5H_2O$ in saturated aqueous $NaHCO_3$ (4 mL) and stirred vigorously until both layers became clear. The organic layer was washed with saturated aqueous $NaHCO_3$, H_2O and brine, dried (Na_2SO_4), filtrated, and concentrated *in vacuo* to give, after purification by preparative TLC (CH_2Cl_2/THF, 85:15), **115** as a white foam (54.8 mg, 78.3%).

^{1}H-NMR ($CDCl_3$): 1.47 (s, 18H, 2x Boc), 1.45-1.75 (m, 4H, kArg γ- and δ-H), 1.75-2.30 (m, 4H, Pro β- and γ-H), 3.16 (d, *J*=5.8, 2H, Phe β-H), 3.30-3.65 (m, 4H, kArg ε-H, Pro δ-H), 3.57 (m, 1H, Pro δ-H), 3.74 (s, 3H, OCH_3), 4.33 (m, 1H, Pro α-H), 4.60 (m, 2H, $OC\underline{H}_2CH{=}CH_2$), 4.75 (m, 1H, Phe α-H), 5.11-5.35 (m, 2H, kArg β-H, $OCH_2CH{=}C\underline{H}_2$), 5.89 (m, 1H, $OCH_2C\underline{H}{=}CH_2$), 7.07-7.17 (m, 2H, Phe aryl), 7.20-7.49 (m, 5H, Phe aryl and α-NH, kArg β-NH), 8.33 (bt, *J*=5.0, 1H, kArg ε-NH) and 11.47

(bs, 1H, kArg ω'-NH). ^{13}C-NMR ($CDCl_3$): 25.3 (δ-kArg), 28.0 ($C(\underline{C}H_3)_3$), 28.2 ($C(\underline{C}H_3)_3$), 28.3 (γ-kArg), 29.6 (γ-Pro b), 30.8 (β-Pro b), 37.8 (β-Phe), 40.2 (ε-kArg), 47.1 (δ-Pro, b), 52.5 (OCH_3), 53.2 (α-Phe), 54.0 (β-kArg), 60.2 (α-Pro b), 66.2 ($O\underline{C}H_2CH=CH_2$), 79.3 ($O\underline{C}(CH_3)_3$), 83.1 ($O\underline{C}(CH_3)_3$), 117.6 ($OCH_2CH=\underline{C}H_2$), 127.3 (aryl-4), 128.7 (aryl-2), 129.2 (aryl-3), 132.6 ($OCH_2\underline{C}H=CH_2$), 135.1 (aryl-1), 153.1 (C(O)O, Boc), 156.1 (C(O)O, Aloc), 158.4 (C=N), 163.4 (C(O)O, Boc), 170.7 ($\underline{C}(O)OCH_3$), 171.7 (C(O)NH), 172.2 (C(O)NH) and 194.7 (α-kArg).

5.6. References and Notes

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Also the presence of starting material and/or by-products may be responsible for the observed explosions. For this reasons the reagent is not commercially available anymore. Recently improved procedures for the preparation of periodinane **114** were published,[b,c] with which the periodinane can be prepared fast and save without hazardous by-products. a) Plumb, J.B.; Harper, D.J. *Chem. Eng. News* **1990**, July 16, 3. b) Ireland; Liu, L. *J. Org. Chem.* **1993**, *58*, 2899. c) Meyer, S.D.; Schreiber, S.L. *J. Org. Chem.* **1994**, *59*, 7549.

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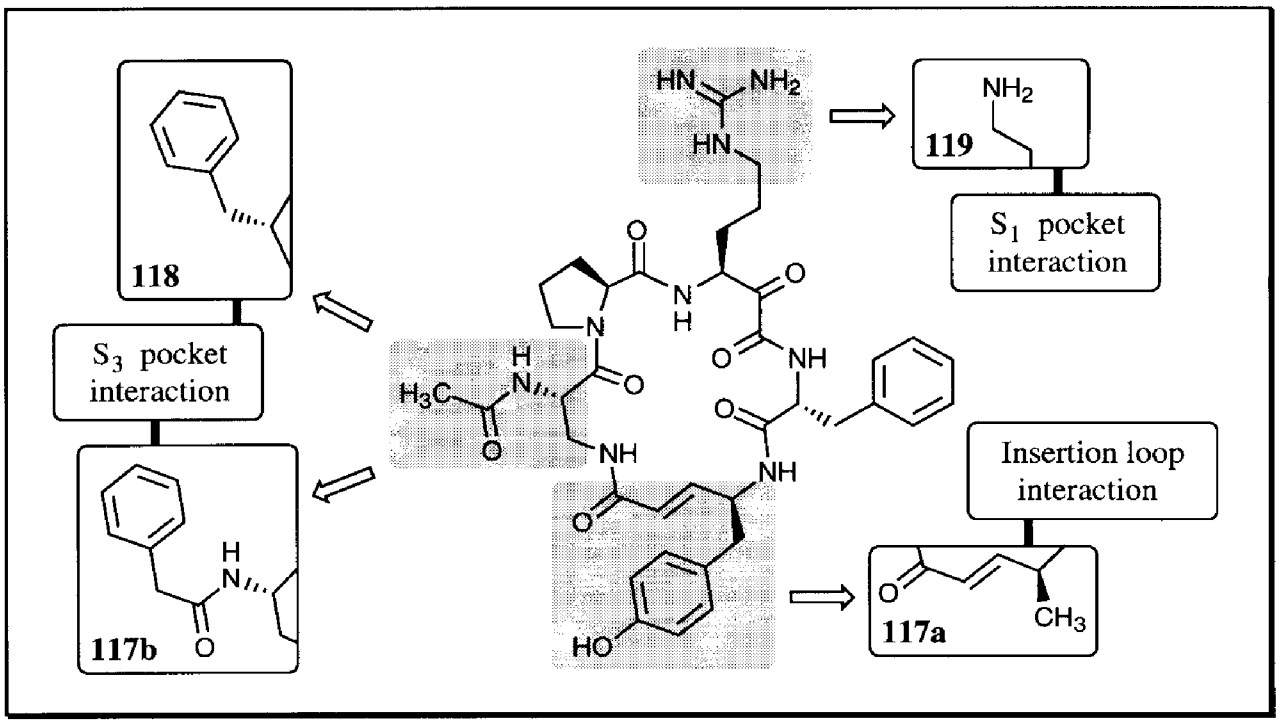
CHAPTER SIX

Synthesis of Cyclotheonamide Analogues



Abstract

The flexible [3 + 2] fragment-condensation approach developed for the synthesis of Cyclotheonamide B was successfully applied to the preparation of four analogues.

To study the role of the hydroxyphenyl group, which was reported to contribute to the binding to thrombin, analogue **117a**, in which this group is replaced by a methyl group, was prepared.

In an attempt to utilize the unoccupied, large hydrophobic S_3 pocket in thrombin a phenyl group was introduced, yielding analogues **117b** and **118**.

Furthermore, in an attempt to improve the pharmacokinetic properties of the natural product, the guanidino moiety was replaced by an aminomethyl group to give **119**.

6.1. Introduction

Our main interest in the properties of Cyclotheonamide lies in its ability to inhibit thrombin. However, Cyclotheonamide also inhibits several related serine proteases very potently; *e.g.* trypsin is inhibited 5 to 10 times more effectively than thrombin (for a discussion of biological data, see Chapter 7).[1] Since the solid-state structures of both the Cyclotheonamide-thrombin complex[2-4] and the Cyclotheonamide-trypsin complex[5] were available, we envisioned that, by perusal of these X-ray data, we would be able to suggest modifications of the natural product's molecular structure resulting in more potent and selective thrombin inhibition. However, comparison of the solid-state structures of the Cyclotheonamide-thrombin and Cyclotheonamide-trypsin complexes, reveal many similarities with respect to enzyme-inhibitor interactions and steric fit, and therefore, fail to explain the greater effectiveness of Cyclotheonamide for inhibition of trypsin relative to thrombin. Thus, no clear proposals for modification towards more selective inhibitors could be put forward on the basis of these data.

The structural features of the 19-membered macrocyclic peptide-mimic which seem to be essential for interaction with thrombin are confined in the Pro–kArg–Phe segment, in which the Pro-Arg motif correlates with the P_1-P_2 positions in the D-Phe–Pro–Arg class of thrombin inhibitors. Furthermore, the α-keto amide is engaged in a covalent interaction (hemiketal) with Ser-195 of the catalytic triad of the enzyme. This hemiketal is stabilized by an intricate hydrogen-bonding network to Ser-195, Gly-193 and His-57. Finally, the D-Phe residue of Cyclotheonamide resides in a hydrophobic region, *i.e.* the assumed S'_2 specificity pocket. An elaborate discussion of the 3-D structure of thrombin and its interactions with substrates and inhibitors is given in Chapter One.

Inspection of the X-ray data of the Cyclotheonamide–human-α-thrombin–hirugen complex suggests that the vTyr-Dpr segment has no strong interaction with the enzyme. However, Maryanoff *et al.* assume an interaction of the hydroxyphenyl group of the vTyr residue with Trp-60D of the insertion loop.[2-4] In our opinion, this interaction is rather obscure and of negligible importance for the overall ligand-enzyme interaction. In order to clarify the role of the hydroxyphenyl group in the interaction of Cyclotheonamide with thrombin we decided to prepare analogue **117a**, in which this group is replaced by a methyl group [Figure 6.1].

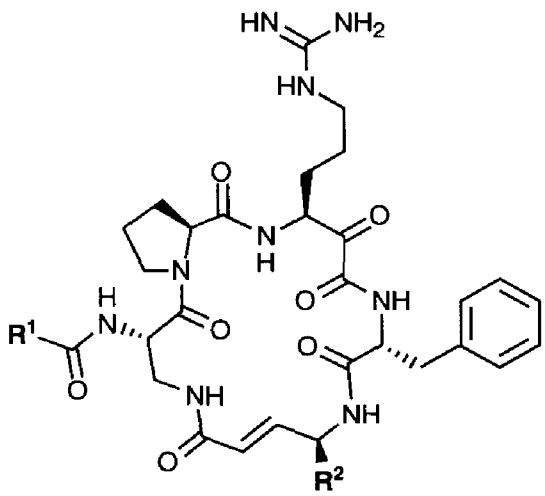
The hydrophilic formamide or acetamide part of Dpr clearly does not satisfy the large hydrophobic S_3 specificity pocket occupied by the D-Phe residue in PPACK and in related D-Phe–Pro–Arg inhibitors [Chapter 1.3.2]. In an attempt to optimize the ligand-enzyme interactions, and consequently to enhance the thrombin inhibition, we decided to replace the Dpr residue of Cyclotheonamide by a D-Phe unit to give analogue **118** with the classical D-Phe–Pro–Arg motif incorporated into the macrocyclic ring system [Figure 6.1]. It should be noted that the ring size in **118** is 18 atoms. A profound S_3-P_3 interaction might also be achieved by simply replacing the acetyl group of the Dpr residue with a more hydrophobic group. Thus, we decided to prepare also cyclopentapeptide **117b** in which the acetyl group is replaced by a phenylacetyl (Pac) group. Furthermore, in this analogue also the vTyr unit is replaced by a vinylogous alanine (vAla).

Also, modifications of the Pro–kArg–D-Phe sequence were considered. Thrombin is selective for substrates containing a basic residue at P_1, *e.g.* arginine and lysine. However, in general, arginine-containing inhibitors have poor pharmacokinetic properties. We therefore prepared **119**, in which the guanidinopropyl moiety (Arg) has been replaced by an aminobutyl group (Lys) [Figure 6.1].

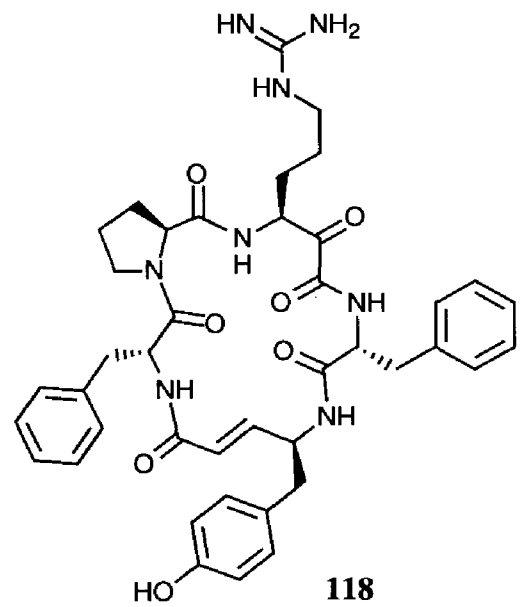
Moreover, this modification might also overcome the frequently encountered, undesirable hypotensive activity of guanidino-containing structures.

At the onset of our investigations, we speculated that the keto amide unit might be involved in a hemiketal complex with thrombin. Therefore, we started the synthesis of des-oxo analogue **120** [Figure 6.1]. However, before the synthesis of **120** was completed, X-ray studies of Maryanoff[2-4] and Schreiber[5] unequivocally established the role of the keto amide unit for the inhibitory activity; this keto group indeed forms a covalent bond (hemiketal) with the Ser-195 of the enzyme [Chapter 1.4.2]. We therefore stopped our efforts towards the preparation of **120** at the stage of a β-homoarginine-containing tripeptide. The synthesis of this tripeptide, together with a new and general method for the preparation of β-homoarginine-containing dipeptides is described in the Appendix.

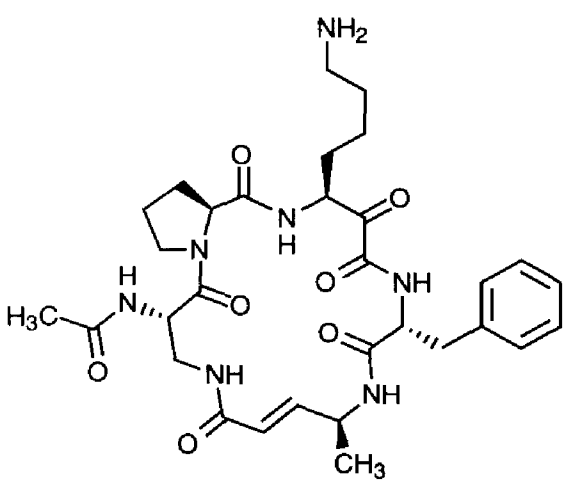
Figure 6.1. Cyclotheonamide (**1b**) and analogues **117a**, **117b**, **118**, **119** and **120**.



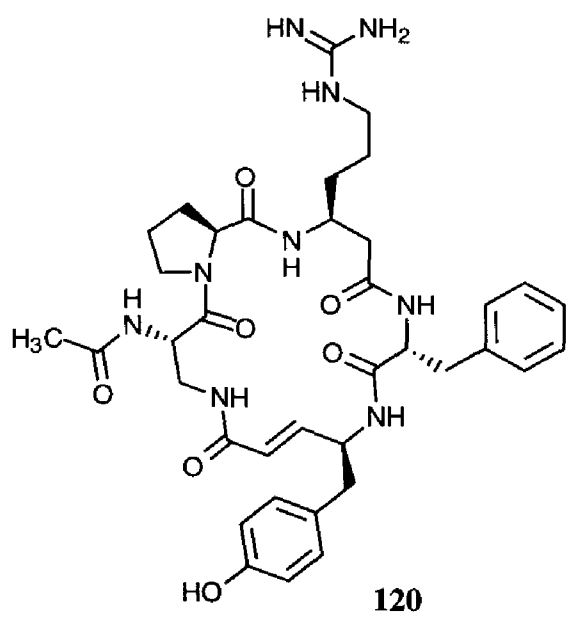
1b: $R^1 = CH_3$, R^2 = 4-hydroxybenzyl
117a: $R^1 = CH_3$, $R^2 = CH_3$
117b: R^1 = Bzl, $R^2 = CH_3$



118



119



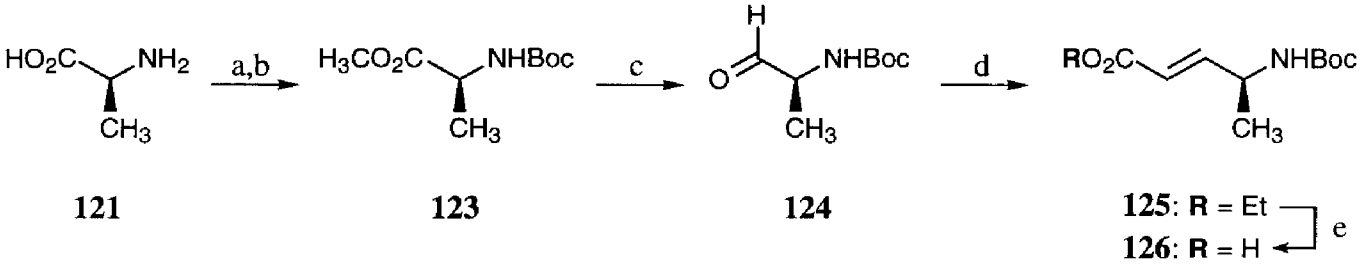
120

6.2. Modification of the vTyr–Dpr Dipeptide Part: Cyclotheonamide Analogues 117a,b

The synthesis of analogues **117a** and **117b** according to our [3 + 2] fragment-condensation approach required the preparation of two new dipeptides, *viz.* **130a** and **130b**, both containing a vinylogous alanine unit [Scheme 6.3].

The vinylogous alanine derivative **126** [Scheme 6.1] was easily prepared by the procedure developed for the vinylogous tyrosine part of Cyclotheonamide B [Chapter 4.3.1]. Thus, *N*-Boc alanine methyl ester (**123**), prepared in two steps from L-alanine (**121**), was converted to aldehyde **124** and reacted immediately with the ylid from triethyl phosphonoacetate to give vinylogous alanine ethyl ester **125**, exclusively having the *E* geometry, in 68% yield from **123**. Hydrolysis of the ethyl ester furnished acid **126** as a colourless solid.

Scheme 6.1. Preparation of vinylogous alanine **126**.

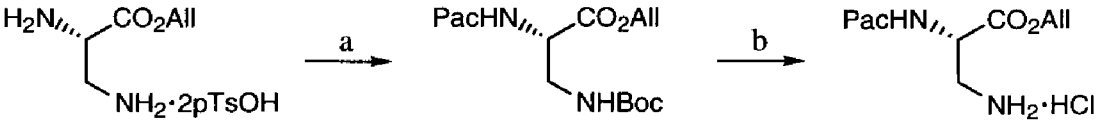


121 **123** **124**

a) $SOCl_2$, MeOH, rt, 2h, reflux, 0.5 h, 95%; b) Boc_2O/TEA, DMF, 50 °C, 45 min, 90%; c) DiBAH, CH_2Cl_2 –68 °C, 15 min, 90%; d) triethyl phosphonoacetate/NaH, THF, –50 → 10 °C, 2.5 h, 76%; e) NaOH, dioxane/H_2O, 16 h, 94%.

Allyl N^{α}-Pac-2,3-diaminopropanoate (**128**) [Scheme 6.2] required for the synthesis of **130b** was prepared from **103** according to the same procedure as was used for the N^{α}-acetyl derivative **106** [Chapter 4.3.2], by using phenylacetyl chloride instead of acetyl chloride.

Scheme 6.2. Preparation of allyl N^{α}-Pac-2,3-diaminopropanoate **128**.

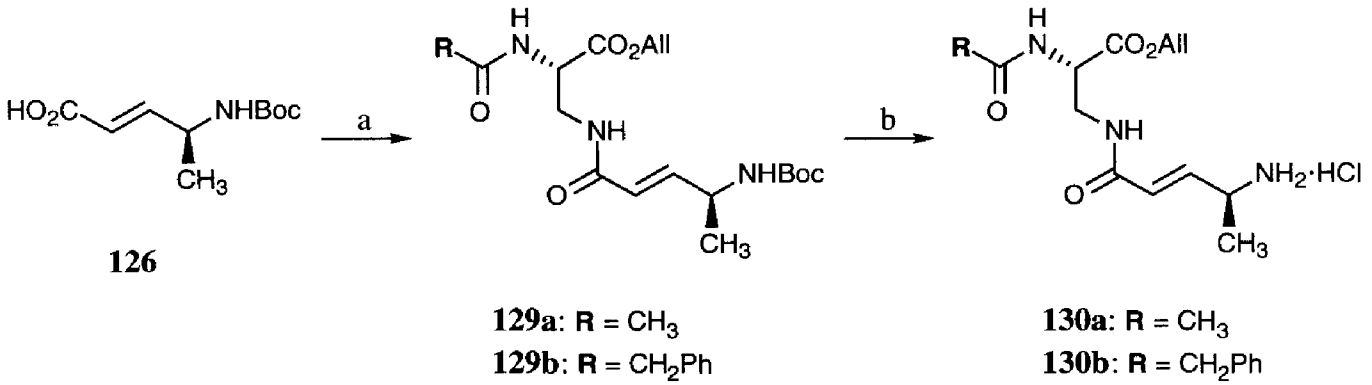


103 **127** **128**

a) Boc_2O/TEA, CH_2Cl_2, –68 → 2 °C, 4 h, phenylacetyl chloride, 2 → 20 °C, 16 h, 55%; b) 3M HCl, Et_2O, 0 °C → rt, 1 h, 82%.

TBTU-coupling of **126** with diaminopropanoic acid derivative **106** or **128** afforded in high yield the protected dipeptides **129a** and **129b**, respectively [Scheme 6.3]. Treatment of these dipeptides with an etheral solution of hydrogen chloride to cleave the *N*-Boc group gave dipeptides **130a** and **130b**, ready for fragment condensation.

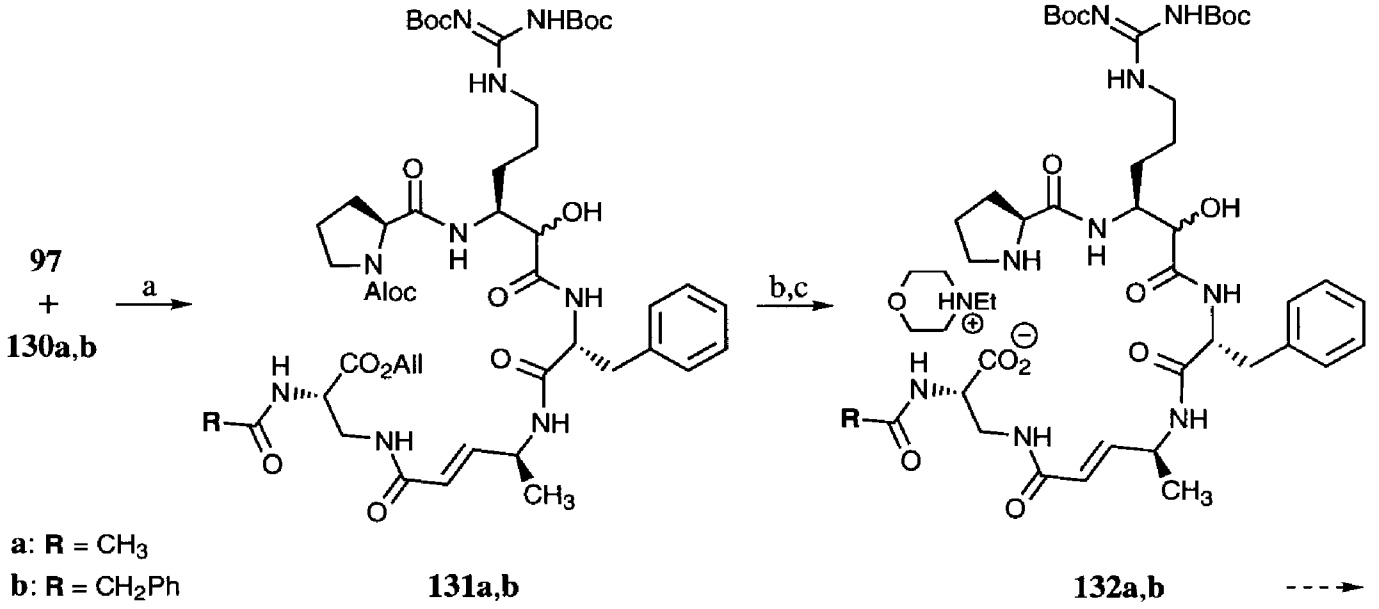
Scheme 6.3. Synthesis of the vAla–Dpr dipeptides **130a,b**.



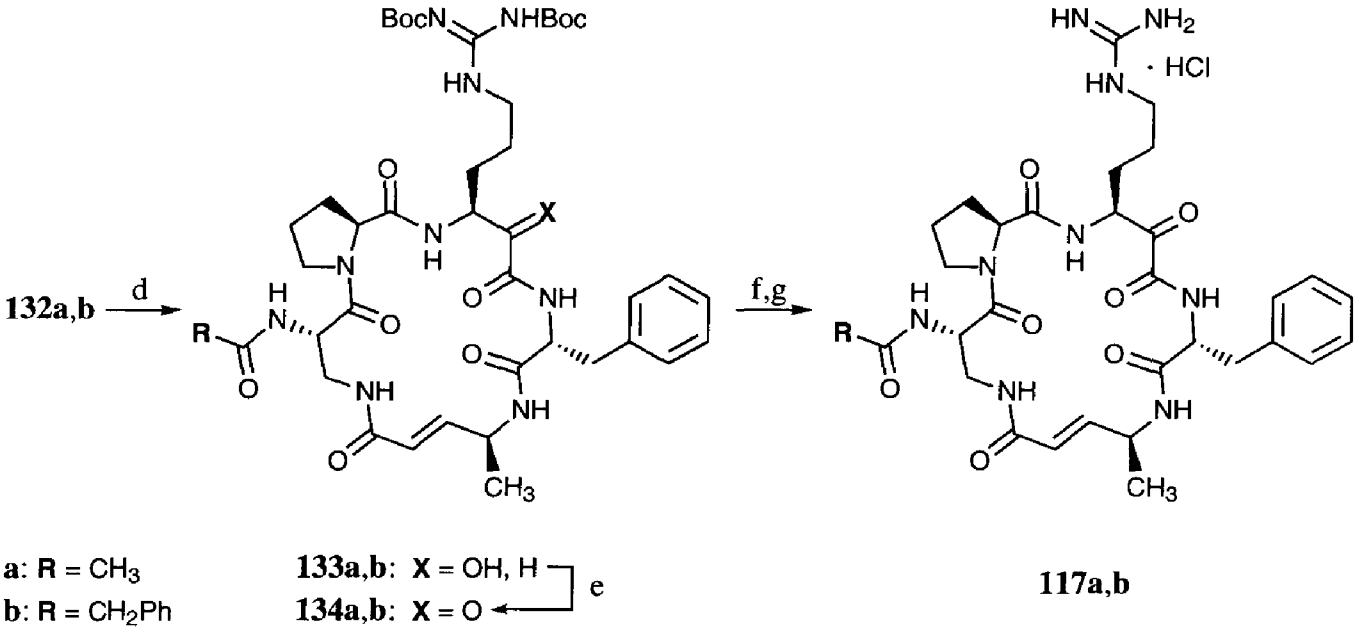
a) **129a**: **106**/TBTU/DiPEA, CH_2Cl_2, 2 h, 88%; **129b**: **128**/TBTU/DiPEA, CH_2Cl_2, 2 h, 99% ; b) **130a,b**: HCl/Et_2O (3M), 0 °C→rt, 1 h, 97 and 100%, respectively.

Elaboration of **130a** and **130b** into the analogues **117a** and **117b**, respectively, *via* the approach developed for Cyclotheonamide B is depicted in Scheme 6.4. The linear pentapeptides **131a,b** were prepared by TBTU-coupling of tripeptide **97** with the corresponding dipeptides **130a,b**.
Deprotection of the termini of **131a,b**, using $Pd(PPh_3)_4$/morpholine, proceeded smoothly and gave the morpholine-salts of the pentapeptides. Purification by preparative TLC (CH_2Cl_2/MeOH/NEM) afforded the *N*-ethyl morpholine salts **132a,b**, which were treated under dilution conditions with TBTU/HOBt/DMAP to give cyclopentapeptides **133a,b**. Surprisingly, the yield of **133a** was only 15% and could not be improved in further experiments. The yield of **133b** (58%) was comparable with that observed in the synthesis of Cyclotheonamide (61%) [Chapter 5.2.3].
Dess-Martin oxidation of **133a,b** (using *t*-butanol, 40 °C, 24 h) and final deprotection followed by purification by HPLC, furnished **117a** and **117b** as white amorphous solids. These cyclopentapeptides were fully characterized by 1D and 2D NMR-techniques and FAB-MS.

Scheme 6.4. Synthesis of cyclopentapeptides **117a** and **117b** from the key intermediates.



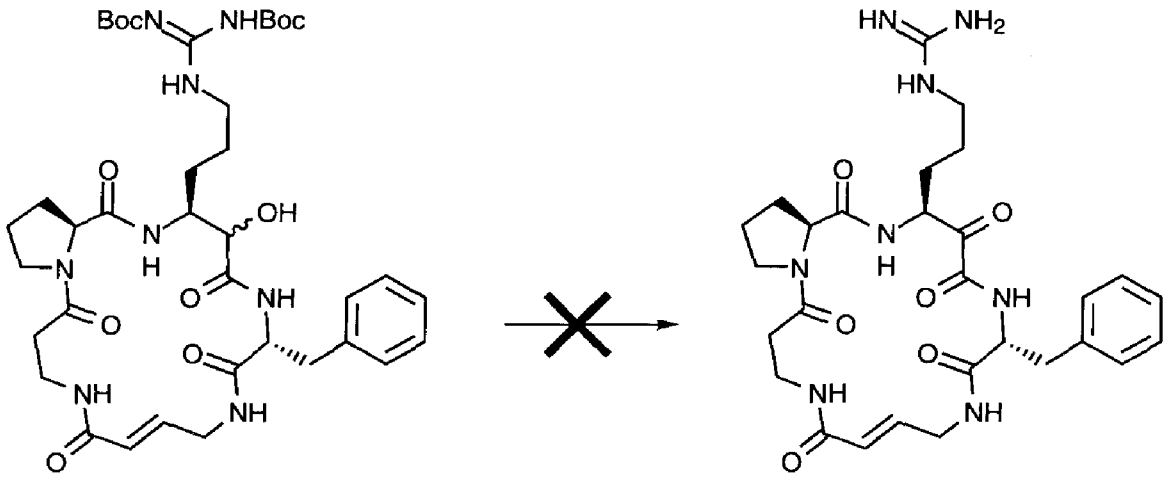
Scheme 6.4. Continued



a) TBTU/DiPEA, 2 h, **131a,b**: 87, 86%; b) $Pd(PPh_3)_4$/morpholine, THF, 45 min; c) preparative TLC on SiO_2, CH_2Cl_2/MeOH/NEM, **132a,b**: 76, 79%; d) TBTU/HOBt/DMAP, CH_2Cl_2 (0.5 mM), 18 h, **133a,b**: 15, 58%; e) Dess-Martin periodinane/*t*-BuOH, CH_2Cl_2/MeCN, 40 °C, 24 h; f) TFA/thioanisole, 115 min; g) HPLC **117a,b**: 59, 55% (3 steps).

Attempts to prepare another Cyclotheonamide analogue, *i.e.* **136** [Scheme 6.5], with a vinylogous glycine unit instead of the vTyr unit were unsuccessful. The protected cyclopentapeptide **135** was obtained in a similar fashion as described for the synthesis of **133a,b** (albeit with much lower yields). However, attempts to convert **135** into **136** failed. This failure, for which we have no good explanation, might be due to the reactivity of the unsubstituted α,β unsaturated amide which might be enhanced upon ring closure of the corresponding linear peptide and/or oxidation of the hydroxy amide group.

Scheme 6.5. Attempted synthesis of analogue **136**.

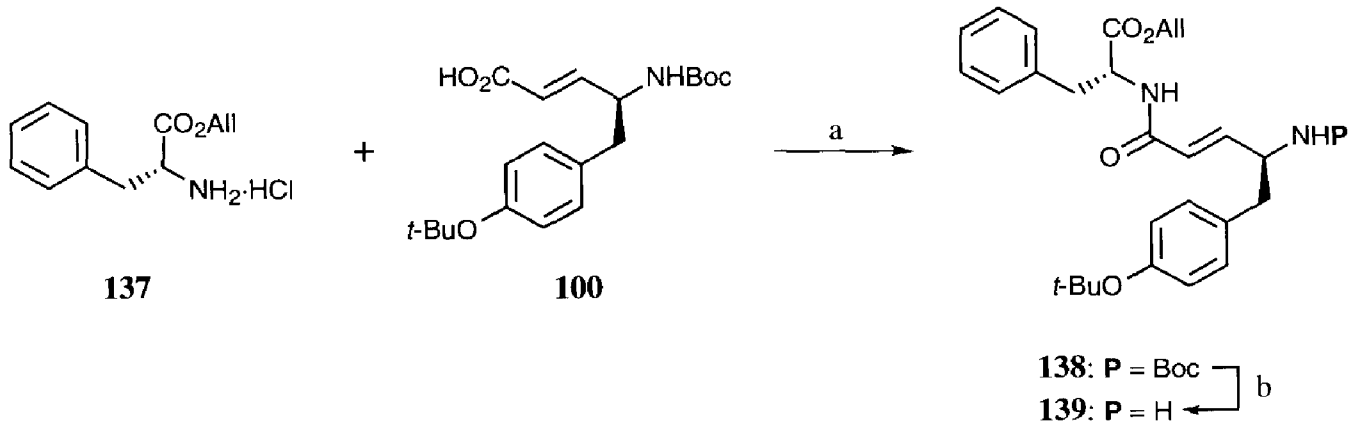


135 **136**

6.3. A D-Phe–Pro–Arg Based Cyclopentapeptide: Cyclotheonamide Analogue 118

For the synthesis of analogue **118**, tripeptide **97** and dipeptide derivative **139** were required [Scheme 6.6]. The preparation of the dipeptide was rather straightforward as it is composed of vinylogous tyrosine **100** [Chapter 4.3.1] and the easily accessible D-phenylalanine allyl ester (**137**). Coupling of the two amino acid derivatives using the TBTU-method yielded the fully protected dipeptide **138** in 92% yield. Selective *N*-Boc deprotection with TMS-triflate/2,6-lutidine furnished the desired dipeptide building block **139**.

Scheme 6.6. Synthesis of dipeptide **139**.



137 **100**

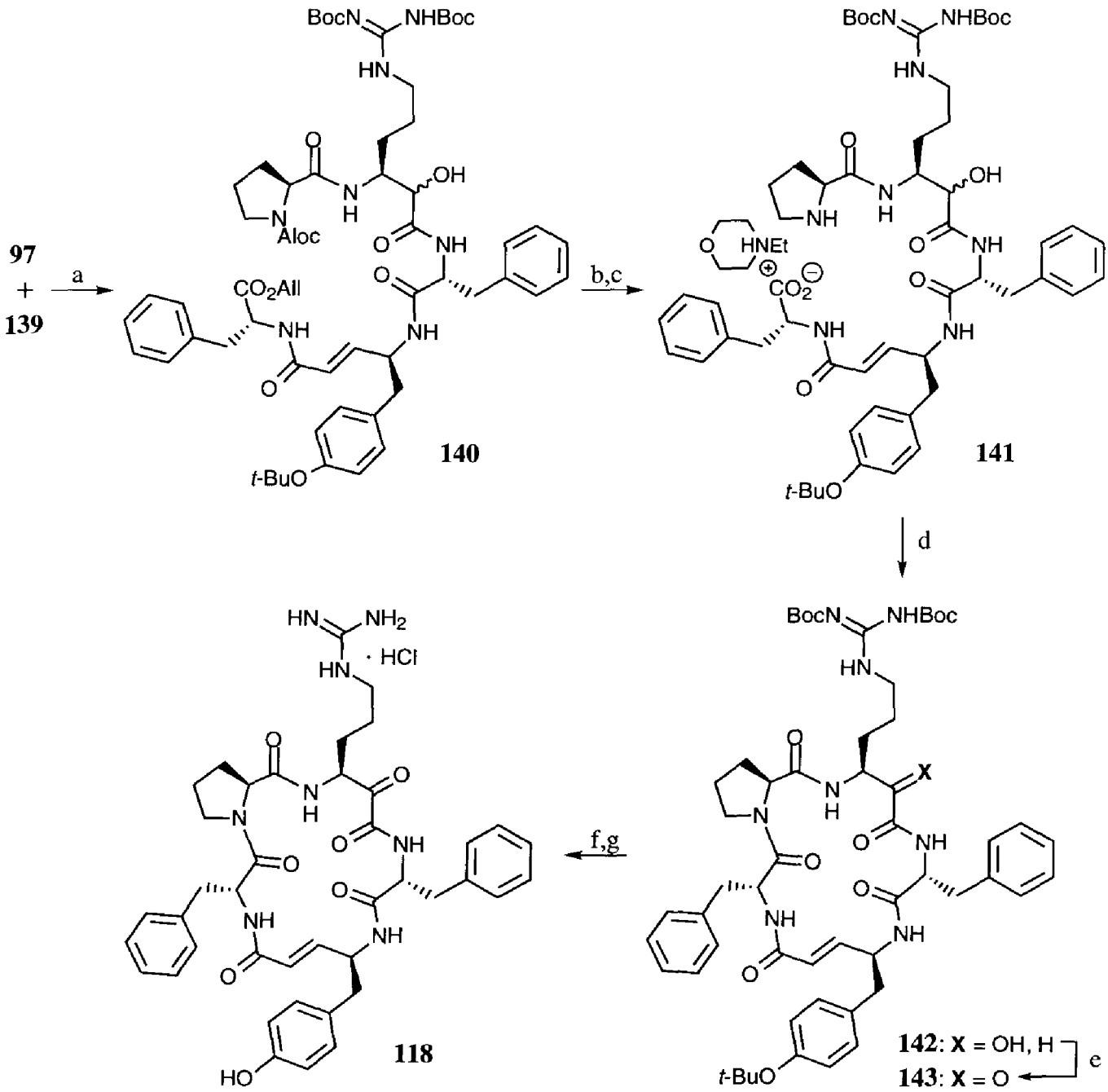
a) TBTU/DiPEA, CH_2Cl_2, 2 h, 89%; b) TMS-triflate (4.1 equiv)/2,6-lutidine (5 equiv), CH_2Cl_2, 0 °C→rt, 2 h, 100%.

Coupling of tripeptide **97** with dipeptide **139** to give pentapeptide **140** proceeded smoothly [Scheme 6.7]. Treatment of **140** with Pd(0) gave the *C,N*-terminal deprotected linear pentapeptide **141** which precipitated from the CH_2Cl_2/MeOH/NEM solution and was collected by centrifugation, as an amorphous white powder. Ring closure followed by oxidation, deprotection, purification by HPLC and lyophilization, yielded a product (≥97.7% pure, according to analytical HPLC) with the correct mass spectrum (FAB-MS). However, the ^{1}H-NMR spectrum in D_2O showed the presence of, at least, two products in a ratio of approximately 7:2, the major one being **118**. The other product was assigned the structure of the linear sequence H-Pro–kArg–D-Phe–vTyr–Dpr(Ac)-OH. HPLC analysis of the NMR sample indicated the formation of several new compounds as four main peaks were observed in a ratio of: 61.6 (**118**), 17.2, 8.7 and 6.9%, respectively.

The formation of the linear pentapeptide is apparently due to ring opening of **118** by hydrolysis of the Pro–Dpr peptide bond. The lesser stability of **118** is presumably caused by a different and more strained ring conformation of the 18-membered ring compared to that of the 19-membered cyclopentapeptides. Comparison of the ^{1}H-NMR spectra of four 19-membered cyclopentapeptides (**1b**, **117a**, **117b** and **119**) with the 18-membered cyclopentapeptide **118** indeed shows a difference in the conformation of the ring, *e.g.* the vicinal coupling constants for H_β–H_γ of the vinylogous amino acid unit in the 19-membered ring ($^3J(H_\beta H_\gamma)$= 2.5 Hz) are smaller than the corresponding coupling constant for the 18-membered ring compound ($^3J(H_\beta H_\gamma)$= 5.6 Hz).
Noteworthy in this context is the observation made during the oxidation reactions. Whereas the

oxidation of the 19-membered cyclopentapeptides **111**, **133a,b** and **157** (*vide infra*) required more than 24 h at 40 °C, the oxidation of **142** was complete within 4.5 h at room temperature. Furthermore, after 24 h reaction time at room temperature, the oxidation product **143** was completely deteriorated, confirming the observation that the 18-membered macrocycle is less stable than the 19-membered ring system.

Scheme 6.7. Synthesis of cyclopentapeptide **118** from the key fragments **97** and **139**.

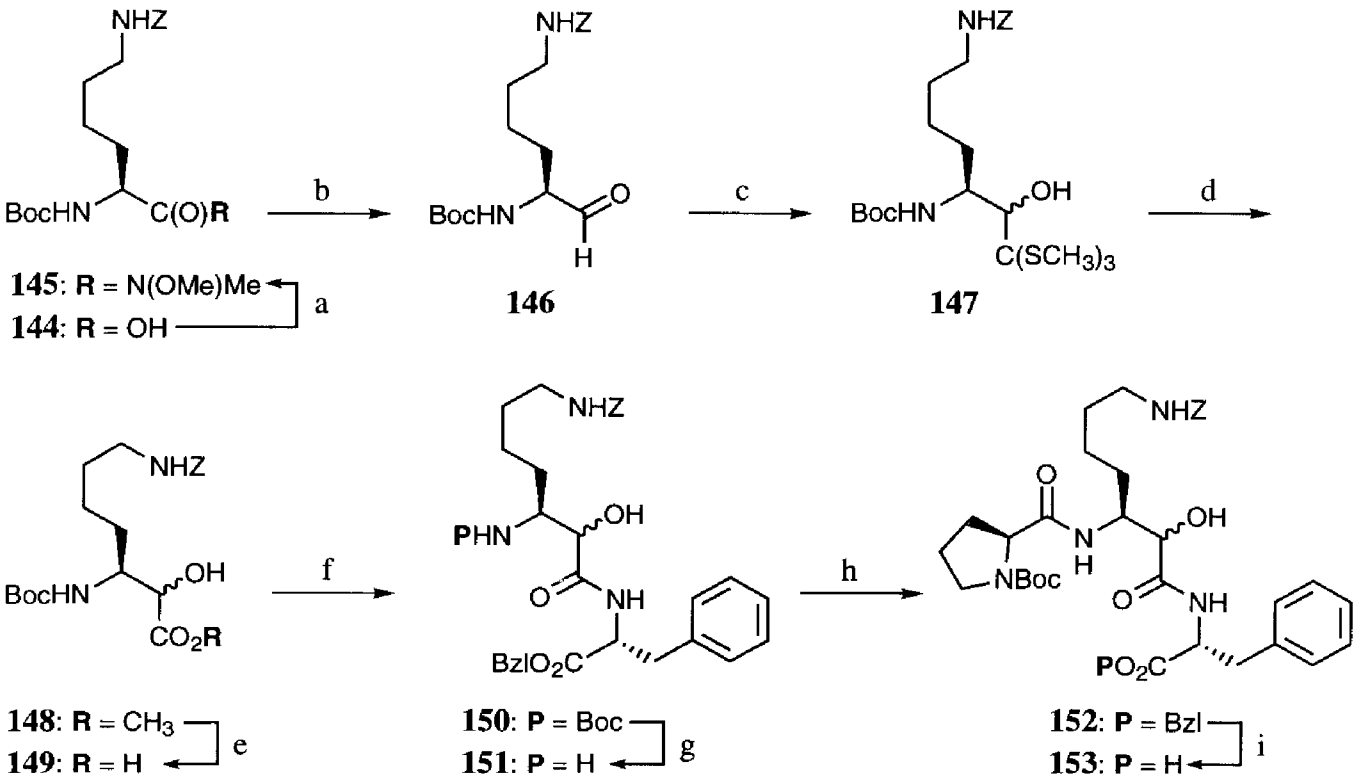


a) TBTU/DiPEA, 2 h, 58%; b) $Pd(PPh_3)_4$/morpholine, THF, 45 min; c) preparative TLC on SiO_2, CH_2Cl_2/MeOH/NEM, 74%; d) TBTU/HOBt/DMAP, CH_2Cl_2 (0.5 mM), 18 h, 69%; e) Dess-Martin periodinane/*t*-BuOH, CH_2Cl_2/MeCN, rt, 3 h; f) TFA/thioanisole, 115 min; g) HPLC: 50% (3 steps).

6.4. Modification of the Arginine Side Chain: Cyclotheonamide Analogue 119

For the synthesis of **119**, the α-hydroxy-β-homolysine-containing tripeptide **153** [Scheme 6.8] was used. The precursor of **153**, *i.e.* the protected tripeptide **152**, had already been prepared several years before, at the start of our project, to explore amino acid homologation by tris(methylthio)methane using protected lysine **144** as a model compound (this method was eventually used for homologation of arginine, Chapter Three). Homologation of **144** to hydroxy ester **148** had already been described by Peet *et al.* but their paper lacked a full experimental procedure.[6] Furthermore, **148** had been used by us to study the elaboration of an α-hydroxy-β-amino acid into a tripeptide.

Scheme 6.8. Synthesis of α-hydroxy-β-homolysine tripeptide **153**.



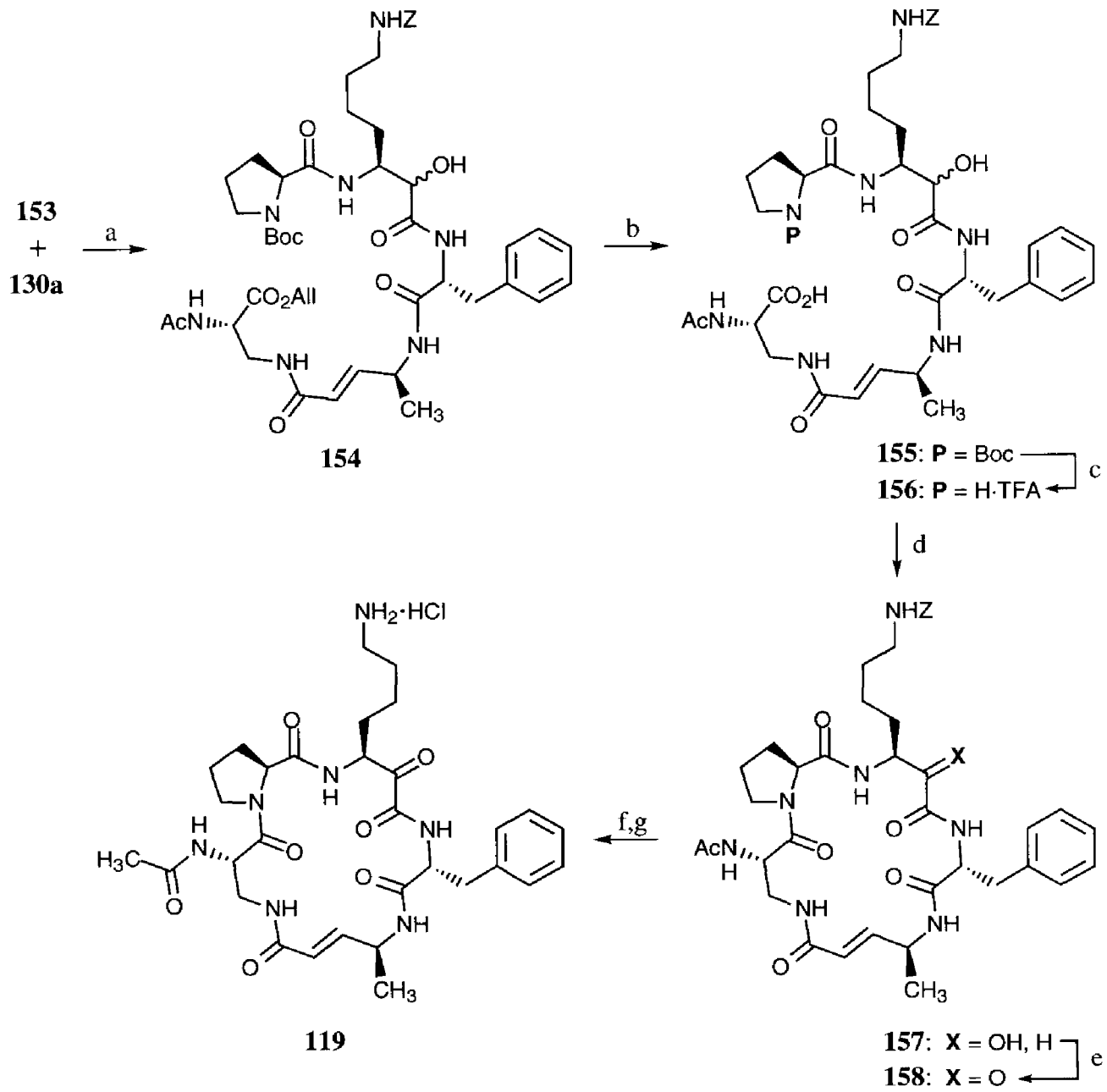
a) DCC/HOBt/*N,O*-dimethylhydroxylamine, CH_2Cl_2, 0 °C, 30 min, rt, 18 h, 75%; b) $LiAlH_4$, Et_2O, –45→5 °C 30 min, 95%; c) $LiC(SCH_3)_3$, THF, –65 °C, 4 h, 70%; d) $HgCl_2/HgO$, $MeOH/H_2O$, 60 °C 6 h, 64%; e) LiOH, $THF/MeOH/H_2O$, 75 min, 90%; f) DCC/HOBt, CH_2Cl_2, 45 min, then D-Phe benzyl ester·HCl/DiPEA, 15 h, 71%; g) TFA/CH_2Cl_2 (3:2), 3 h, 77%; h) *N*-Boc Pro/EDC/HOBt/DiPEA, THF, 21 h, 51%; i) LiOH, $THF/MeOH/H_2O$, 12 min, 52%.

Although the protecting groups of **152** were different from the ones used in our successful [3 + 2] approach, elaboration of this tripeptide into a Cyclotheonamide analogue was without major problems. Thus, selective cleavage of the benzyl ester of **152** was achieved by the *fast hydrolysis* procedure [Chapter 3.4.3], to give **153** in 90% yield after purification by chromatography.

Pentapeptide **154** [Scheme 6.9], obtained by coupling of **153** and **130b** [Scheme 6.3] , was de-allylated by treatment with 20 equiv of morpholine and 5 mol% $Pd(PPh_3)_4$. The resulting product **155** was treated with TFA/CH_2Cl_2 to yield **156**. Macrocyclization of **156** afforded **157** in 33% yield. Subsequent Dess-Martin oxidation, followed by cleavage of the Z group of the homolysine side chain

by treatment with TFA/thioanisole (rt, 3 h) yielded **119**, which was purified by HPLC. This cyclopentapeptide was fully characterized by 1D and 2D NMR-techniques and FAB-MS.

Scheme 6.9. Synthesis of cyclopentapeptide **119** from the key intermediates.



a) TBTU/DiPEA, THF, 2 h, 84%; b) $Pd(PPh_3)_4$/morpholine, THF, 45 min, quantitative; c) TFA/CH_2Cl_2 (3:2), 3 h, quantitative; d) TBTU/HOBt/DMAP, THF (0.5 mM), 18 h, 33%; e) Dess-Martin periodinane/*t*-BuOH, CH_2Cl_2/MeCN, 40 °C, 24 h ; f) TFA/thioanisole, 3 h; g) HPLC, 45% (3 steps).

6.5. Conclusions

The [3 + 2] fragment-condensation approach developed for the total synthesis of Cyclotheonamide B was successfully applied to the preparation of the four analogues **117a,b**, **118** and **119**.

As far as the synthesis of the *C,N*-terminal deprotected linear pentapeptides is concerned, no major differences or difficulties compared to the synthesis of the natural product were encountered. However, the efficiencies of the ring closure varied strikingly [Table 6.1]. Whereas **132b** and **141** (entry 3 and 4) gave the corresponding cyclopeptides in approximately the same yield as was observed for **111** (entry 1) in the synthesis of Cyclotheonamide B, cyclization of **156** (entry 5) proceeded in 33%, and **132a** (entry 2) gave only 15% of the desired product. Furthermore, ring closure of a sixth pentapeptide (**159**, entry 6), containing a vinylogous glycine moiety also resulted in a poor yield (12%) of the corresponding macrocycle.

Table 6.1. Yields of cyclization of the linear pentapeptides.

	linear pentapeptide sequence (without protecting groups)	number of aromatic groups	cyclopeptide	yield (%)
1	Pro–hArg–D-Phe–vTyr–Dpr(Ac) (**111**)	2	**112**	61
2	Pro–hArg–D-Phe–vAla–Dpr(Ac) (**132a**)	1	**133a**	15
3	Pro–hArg–D-Phe–vAla–Dpr(Pac) (**132b**)	2	**133b**	58
4	Pro–hArg–D-Phe–vTyr–D-Phe (**141**)	3	**142**	69
5	Pro–hLys–D-Phe–vAla–Dpr(Ac) (**156**)	2	**157**	33
6	Pro–hArg–D-Phe–vGly–β-Ala (**159**)	1	**135**	12

It appears that the efficiency of the ring closure depends strongly on the molecular structure of the linear peptide. The turn-stabilizing amino acids (*i.e.* Pro and D-Phe, see Chapter 5.2) are present in all of the six linear pentapeptides. Therefore, it must be concluded that the presence of these turn-stabilizing amino acids does not have a profound influence on the efficiency of the ring closure and that the success of the cyclization is governed by other structural features.
In comparing the structure of the six linear pentapeptides it is noticeable that **141**, having three aromatic amino acid residues, gave the highest yield of cyclization product, whereas **132a** and **159**, with only one aromatic residue, gave the poorest results. Two of the three pentapeptides with two aromatic groups, *i.e.* **111**, **132b**, gave also reasonable yields, whereas **156** (with a benzyloxycarbonyl group at the lysine side chain) gave a much lower yield. From these findings it might be concluded that folding of the linear pentapeptide by intramolecular aromatic interactions (hydrophobic collapse) is of vital importance for the efficiency of the cyclization reaction.

A second remarkable feature is the decreased stability of the 18-membered ring analogue **118** and its precursor **143** compared to the 19-membered macrocycles. Although no exact explanation can be given at this moment, we anticipate that the smaller ring size of this 18-membered macrocycle results in more strain, which leads to a decreased stability.

Finally, it can be concluded that our [3 + 2] fragment-condensation approach offers a fast and flexible protocol for the preparation of Cyclotheonamide analogues. The biological evaluation of the analogues presented in this chapter is reported in Chapter Seven.

6.6. Experimental

Detailed general experimental information is given in Section 3.6.

Methyl 2(*S*)-aminopropanoate hydrochloride (122)

$SOCl_2$ (19.0 mL) was cautiously added to MeOH (120 mL) at –15 °C. Subsequently, L-alanine (14.9 g, 167 mmol) was added and the resulting suspension was stirred at room temperature for 2 h. After an additional 30 min at reflux a clear solution was obtained. The volatiles were removed *in vacuo* and the remaining off-white solid was crystallized from MeOH/EtOAc to give **122** as a colourless needles (22.2 g; 95.0%); mp 109-110 °C.

^{1}H-NMR (DMSO-d_6): 1.45 (d, J=6.7, 3H, CH_3), 3.72 (s, 3H, OCH_3), 4.08 (q, J=6.7, 1H, α-H) and 8.73 (bs, 3H, NH_3^+).

Methyl 2(*S*)-[(*tert*-butyloxycarbonyl)amino]propanoate (123)

To a stirred solution of **122** (5.84 g, 41.8 mmol) and TEA (15.0 mL) in DMF (110 mL) at 63 °C was added Boc_2O (10.0 g, 45.8 mmol). After 30 min the turbid reaction mixture was allowed to cool to room temperature, and the solvent was evaporated *in vacuo*. The residue was partitioned between CH_2Cl_2 and H_2O, the layers were separated, and the organic layer was sequentially washed with aqueous $KHSO_4$ (6%, 2x), H_2O and brine, dried (Na_2SO_4), filtrated, and concentrated *in vacuo* to give, after purification by column chromatography (PE/EtOAc, 8:1), **123** as a colourless oil (7.63 g; 89.7%).

^{1}H-NMR ($CDCl_3$): 1.37 (d, J=6.7, 3H, CH_3), 1.42 (s, 9H, Boc), 3.72 (s, 3H, OCH_3), 4.30 (m, 1H, α-H) and 5.02 (m, 1H, α-NH).

2(*S*)-[(*tert*-Butyloxycarbonyl)amino]propanal (124)

To a stirred solution of methyl ester **123** (5.07 g, 24.9 mmol) in CH_2Cl_2 (250 mL) at –65 °C was added DiBAH (1 N in hexanes, 60.0 mL, 60.0 mmol) in 20 min. Subsequently, a solution of EtOH/36% HCl_{aq} (9:1, 5.0 mL) was added slowly (the temperature was kept below –65 °C). After the addition was complete, the reaction mixture was added to a vigorously stirred solution of HCl_{aq} (1 N, 400 mL) at 0 °C. The layers were separated, and the aqueous layer was extracted with ice-cold CH_2Cl_2 (2x). The combined organic layers were washed with ice-cold HCl_{aq} (1 N, 2x), ice-cold H_2O (2x) and ice-cold brine, dried (Na_2SO_4), and concentrated *in vacuo* at ambient temperature, to give aldehyde **124** as a colourless oil (3.90 g; 90.4%).

^{1}H-NMR ($CDCl_3$): 1.35 (d, J=6.7, 3H, CH_3), 1.43 (s, 9H, Boc), 4.25 (m, 1H, α-H), 5.07 (m, 1H, α-NH) and 9.54 (s, 1H, C(O)H).

Ethyl 4(*S*)-[(*tert*-butyloxycarbonyl)amino]pent-2(*E*)-enoate (125)

Prepared from aldehyde **124** (3.90 g, 22.5 mmol), triethyl phosphonoacetate (10.0 mL, 50.0 mmol) and NaH (60% in mineral oil, 2.00 g, 50.0 mmol) following the same procedure as described for the preparation of **99** [Chapter Four]. Purification of the crude product by column chromatography (PE/EtOAc, 4:1) gave **125** as a colourless, clear oil (4.18 g; 76.2%).

^{1}H-NMR ($CDCl_3$): 1.26 (d, J=6.7, 3H, CH_3), 1.28 (t overlapping, 3H, $OCH_2C\underline{H}_3$), 1.45 (s, 9H, Boc), 2.80 (m, 2H, δ-H), 4.18 (q, J=6.7, 2H, $OC\underline{H}_2CH_3$), 4.38 (m, 1H, γ-H), 4.55 (m, 1H, γ-NH), 5.88 (d, J=16.0, 1H, α-H) and 6.88 (dd, J=16.0 and 4.7, 1H, β-H). ^{13}C-NMR ($CDCl_3$): 13.9 ($OCH_2\underline{C}H_3$), 19.9 (δ), 28.1 ($OC(\underline{C}H_3)_3$), 46.7 (γ), 60.0 ($O\underline{C}H_2CH_3$), 79.1 ($O\underline{C}(CH_3)_3$), 119.7 (α), 149.3 (β), 154.7 (C(O)O, Boc) and 166.1 ($\underline{C}(O)OCH_2CH_3$).

4(*S*)-[(*tert*-Butyloxycarbonyl)amino]pent-2(*E*)-enoic acid (126)

Prepared from **125** (2.40 g, 9.86 mmol) and aqueous NaOH (1 N, 10.0 mL) following the same procedure as described for the synthesis of **100** [Chapter Four]. The crude product was crystallized from hexane to give **126** as colourless crystalline solid (1.99 g, 93.5%); mp 89-99 °C

^{1}H-NMR ($CDCl_3$): 1.28 (d, *J*=6.7, 3H, CH_3), 1.43 (s, 9H, Boc), 4.31-4.68 (m, 2H, γ-H, γ-NH), 5.89 (dd, *J*=15.2 and 1.5, 1H, α-H) 6.94 (dd, *J*=15.2 and 4.7, 1H, β-H) and 11.69 (bs, 1H, COOH). ^{13}C-NMR ($CDCl_3$): 20.0 (δ), 28.2 ($OC(\underline{C}H_3)_3$), 47.0 (γ), 77.3 ($O\underline{C}(CH_3)_3$), 79.9 ($O\underline{C}(CH_3)_3$), 119.6 (α), 151.6 (β), 154.9 (C(O)O, Boc) and 171.1 (C(O)OH). FAB-HRMS: calcd for $[C_{10}H_{17}NO_4 + H]^+$ 216.1236, found 216.1242.

Allyl 2(*S*)-phenylacetylamino-3-[(*tert*-butyloxycarbonyl)amino]propanoate (127)

Prepared from **103** (4.20g, 8.60 mmol), TEA (4.77 mL, 34.4 mmol), Boc_2O (1.85 g, 8.48 mmol) and phenylacetyl chloride (2.71 g, 17.5 mmol) according to the one-pot procedure described for **105** [Chapter Four]. The crude product was purified by MPLC (CH_2Cl_2/THF, 96:4), to give **127** as a white amorphous powder (1.72 g, 55.2%); mp 89.5-92 °C

^{1}H-NMR ($CDCl_3$): 1.40 (s, 9H, Boc), 3.49 (m, 2H, β-H), 3.59 (s, 2H, $C\underline{H}_2Ph$), 4.52-4.68 (m, 3H, α-H and $OC\underline{H}_2CH=CH_2$), 4.75 (bt, *J*=6.0, 1H, β-NH), 5.28 (m, 2H, $OCH_2CH=C\underline{H}_2$), 5.85 (m, 1H, $OCH_2-C\underline{H}=CH_2$), 6.64 (bd, *J*=6.7, 1H, α-NH) and 7.19-7.39 (m, 5H, aryl). ^{13}C-NMR ($CDCl_3$): 28.2 ($OC(\underline{C}H_3)_3$), 42.0 (β), 43.4 ($\underline{C}H_2Ph$), 53.9 (α), 66.2 ($O\underline{C}H_2CH=CH_2$), 80.0 ($O\underline{C}(CH_3)_3$), 118.9 ($OCH_2CH=C\underline{H}_2$), 127.2 (aryl-4), 128.8 (aryl-2), 129.3 (aryl-3), 131.3 ($OCH_2\underline{C}H=CH_2$), 134.4 (aryl-1), 156.5 (C(O)O, Boc), 169.8 (C(O)OAll) and 171.2 (C(O)NH, Pac). FAB-HRMS: calcd for $[C_{19}H_{26}N_2O_5 + H]^+$ 363.1920, found 363.1890.

Allyl 2(*S*)-phenylacetylamino-3-aminopropanoate hydrochloride (128)

Prepared from **127** (1.15 g, 3.17 mmol) according to the same procedure as described for synthesis of **106** [Chapter Four]. Recrystallization from EtOAc furnished **128** as a colourless solid (0.78 g; 82.3%); mp 115-117 °C

^{1}H-NMR (DMSO-d_6): 3.19 (m, 2H, β-H), 3.56 (s, 2H, $C\underline{H}_2Ph$), 4.52-4.69 (m, 3H, α-H and $OC\underline{H}_2CH=CH_2$), 5.29 (m, 2H, $OCH_2CH=C\underline{H}_2$), 5.87 (m, 1H, $OCH_2C\underline{H}=CH_2$), 8.26 (bs, 3H, β-NH_3^+), 8.89 (bd, *J*=7.0, 1H, α-NH). ^{13}C-NMR (DMSO-d_6): 38.8 (β), 41.7 ($\underline{C}H_2Ph$), 50.0 (α), 65.3 ($OC\underline{H}_2CH=CH_2$), 117.8 ($OCH_2CH=\underline{C}H_2$), 126.2 (aryl-4), 127.9 (aryl-2), 129.0 (aryl-3), 131.8 ($OCH_2\underline{C}H=CH_2$), 135.5 (aryl-1), 168.6 (C(O)OAll) and 170.7 (C(O)NH, Pac). FAB-HRMS: calcd for $[C_{14}H_{18}N_2O_3 + H]^+$ 263.1396, found 263.1425.

Allyl 2(*S*)-acetylamino-3-[[4(*S*)-[(*tert*-butyloxycarbonyl)amino]pent-2(*E*)-enoyl]amino]propanoate (129a)

Prepared from **119** (0.86 g, 4.00 mmol), **106** (1.02 g, 4.58 mmol), DiPEA (814 μL, 4.67 mmol) and TBTU (1.59 mL, 9.12 mmol) according to the same procedure as described for the synthesis of **107** [Chapter Four]. Pure **129a** was obtained after aqueous work-up as a white foam (1.35 g, 88.0%).

^{1}H-NMR ($CDCl_3$): 1.25 (d, *J*=6.7, 3H, vAla δ-H), 1.43 (s, 9H, Boc), 2.03 (s, 3H, Ac), 3.73 (bt, *J*=5.6, 2H, Dpr β-H), 4.34 (m, 1H, vAla γ-H), 4.53-4.73 (m, 3H, Dpr α-H and $OC\underline{H}_2CH=CH_2$), 4.84 (m, 1H, vAla γ-NH), 5.30 (m, 2H, $OCH_2CH=C\underline{H}_2$), 5.89 (dd, *J*=15.8, and 1.5, 1H, vAla α-H), 5.89 (m overlapping, 1H, $OCH_2C\underline{H}=CH_2$), 6.73 (dd, *J*=15.8 and 5.3, 1H, vAla β-H), 6.77 (m overlapping, 1H, Dpr β-NH) and 7.05 (m, 1H, Dpr α-NH). ^{13}C-NMR ($CDCl_3$): 20.5 (δ-vAla), 23.0 ($C(O)\underline{C}H_3$), 28.3 ($OC(\underline{C}H_3)_3$), 41.4 (β-Dpr), 47.1 (γ-vAla), 53.5 (α-Dpr), 66.4 ($O\underline{C}H_2CH=CH_2$), 79.7 ($O\underline{C}(CH_3)_3$),

119.0 ($OCH_2CH=\underline{C}H_2$), 121.9 (α-vAla), 131.3 ($OCH_2\underline{C}H=CH_2$), 145.9 (β-vAla), 155.1 (C(O)O, Boc), 166.9 (C(O)NH), 170.1 ($\underline{C}(O)CH_3$) and 170.8 ($\underline{C}(O)OAll$).

Allyl 2(*S*)-phenylacetylamino-3-[[4(*S*)-[(*tert*-butyloxycarbonyl)amino]pent-2(*E*)-enoyl]-amino]propanoate (129b)

Prepared from **119** (0.38 g, 1.77 mmol), **128** (0.55 g, 1.84 mmol), DiPEA (686 μL, 3.94 mmol) and TBTU (0.64 mL, 2.00 mmol) according to the same procedure as described for the preparation of **107** [Chapter Four]. Pure **129b** was obtained after aqueous work-up as a white foam (0.81 g, 99.8%).

^{1}H-NMR ($CDCl_3$): 1.25 (d, *J*=6.7, 3H, vAla δ-H), 1.44 (s, 9H, Boc), 3.59 (s, 2H, $C\underline{H}_2Ph$), 3.67 (m, 2H, Dpr β-H), 4.35 (m, 1H, vAla γ-H), 4.53-4.67 (m, 4H, vAla γ-NH, Dpr α-H and $OC\underline{H}_2CH=CH_2$), 5.29 (m, 2H, $OCH_2CH=C\underline{H}_2$), 5.77 (dd, *J*=15.6 and 1.5, 1H, vAla α-H), 5.85 (m, 1H, $OCH_2-C\underline{H}=CH_2$), 6.25 (m, 1H, Dpr β-NH), 6.66 (dd, *J*=15.8 and 4.7, 1H, vAla β-H), 6.73 (bd, *J*=6.1, 1H, Dpr α-NH) and 7.20-7.49 (m, 5H, aryl).

Allyl 2(*S*)-acetylamino-3-[[4(*S*)-aminopent-2(*E*)-enoyl]amino]propanoate·HCl (130a)

Prepared from **129a** (1.35 g, 3.52 mmol) according to the same procedure as described for the synthesis of **106** [Chapter Four]. Compound **130a** was obtained as a white powder (1.09 g, 96.8%).

^{1}H-NMR (DMSO-d_6): 1.31 (d, *J*=6.7, 3H, vAla δ-H), 1.87 (s, 3H, Ac), 3.48 (m, 2H, Dpr β-H), 3.96 (m, 1H, vAla γ-H), 4.33 (bq, *J*=6.1, 1H, Dpr α-H), 4.55 (bd, *J*=5.0, 2H, $OC\underline{H}_2CH=CH_2$), 5.26 (m, 2H, $OCH_2CH=C\underline{H}_2$), 5.89 (m, 1H, $OCH_2C\underline{H}=CH_2$), 6.13 (d, *J*=15.2, 1H, vAla α-H), 6.64 (dd, *J*=15.2 and 6.1, 1H, vAla β-H), 8.40 (bs, 3H, NH_3^+), 8.49 (bd overlapping, *J*=7.0, 1H, Dpr α-NH) and 8.58 (bt, *J*=5.7, 1H, Dpr β-NH).

Allyl 2(*S*)-phenylacetylamino-3-[[4(*S*)-aminopent-2(*E*)-enoyl]amino]propanoate·HCl (130b)

Prepared from **129b** (0.73 g, 1.59 mmol) according to the same procedure as described for the synthesis of **106** [Chapter Four]. Compound **130b** was obtained as a white powder (0.63 g, 100%).

^{1}H-NMR (DMSO-d_6): 1.30 (d, *J*=6.7, 3H, vAla δ-H), 3.38-3.67 (m, 4H, Dpr β-H and $C\underline{H}_2Ph$), 3.98 (m, 1H, vAla γ-H), 4.40 (bq, *J*=6.4, 1H, Dpr α-H), 4.53 (bd, *J*=4.8, 2H, $OC\underline{H}_2CH=CH_2$), 5.25 (m, 2H, $OCH_2CH=C\underline{H}_2$), 5.87 (m, 1H, $OCH_2C\underline{H}=CH_2$), 6.12 (d, *J*=15.2, 1H, vAla α-H), 6.65 (dd, *J*=6.1 and 15.2, 1H, vAla β-H), 7.15-7.45 (m, 5H, aryl), 8.31 (bs, 3H, NH_3^+), 8.57 (bt, *J*=5.7, 1H, Dpr β-NH) and 8.71 (bd, *J*=7.1, 1H, Dpr α-NH).

Allyl 2(*S*)-acetylamino-3-[[4(*S*)-[[2(*R*)-[[2(*R,S*)-hydroxy-3(*S*)-[[[1-(allyloxycarbonyl)pyrrolidin-2(*S*)-yl]carbonyl]amino]-6-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]-methyl]amino]hexanoyl]amino]-3-phenylpropanoyl]amino]pent-2(*E*)-enoyl]amino]propanoate (131a)

Prepared from tripeptide **97** (563 mg, 0.769 mmol), dipeptide **130a** (320 mg, 1.00 mmol), DiPEA (350 μL, 2.00 mmol) and TBTU (321 mg, 1.00 mmol) according to the same procedure as described for the synthesis of **110** [Chapter Four]. After aqueous work-up, the product was purified by preparative TLC (CH_2Cl_2/THF/EtOH, 120:80:1.5), to give **131a** as a white foam (680 mg, 88.6%).

^{1}H-NMR (DMSO-d_6): 1.00 (d, *J*=6.7, 3H, vAla δ-H), 1.07-1.55 (m, 4H, hArg γ- and δ-H), 1.38 (s, 9H, Boc), 1.47 (s, 9H, Boc), 1.61-1.89 (m, 3H, Pro β- and γ-H), 1.82 (s, 3H, Ac), 2.00 (m, 1H, Pro β-H), 2.88 (bd, *J*=6.0, 2H, Phe β-H), 3.13-3.45 (m, 5H, Pro δ-H, hArg ε-H, Dpr β-H), 3.51 (m, 1H, Dpr β-H), 3.86 (m, 1H, hArg α-H), 3.99 (m, 1H, hArg β-H), 4.13-4.51 (m, 8H, Pro α-H, Phe α-H, vAla γ-H, Dpr α-H and 2x $OC\underline{H}_2CH=CH_2$), 5.07-5.38 (m, 4H, 2x $OCH_2CH=C\underline{H}_2$), 5.70-5.99 (m, 4H, hArg OH, vAla α-H and 2x $OCH_2C\underline{H}=CH_2$), 6.52 (dd, *J*=15.2 and 1.5, 1H, vAla β-H), 7.10-7.31 (m, 5H, Phe aryl),

7.54-7.72 (m, 2H, hArg β-NH, Phe α-NH), 8.10-8.25 (m, 3H, hArg ε-NH, vAla γ-NH, Dpr β-NH), 8.29 (d, *J*= 8.4, 1H, Dpr α-NH) and 11.50 (bs, 1H, hArg ω-NH). ^{13}C-NMR ($CDCl_3$): 20.0 (δ-vAla), 22.5 (C(O)CH_3), 24.3 (δ-hArg), 25.6 (γ-hArg), 27.7 (OC(CH_3)$_3$), 28.0 (OC(CH_3)$_3$), 29.2 (γ-Pro, b), 30.8 (β-Pro, b), 38.6 (β-Phe), 40.4 (β-Dpr), 40.4 (ε-hArg), 45.3 (γ-vAla), 46.8 (δ-Pro, b), 51.3 (α-Phe), 52.8 (β-hArg), 53.9 (α-Dpr), 60.6 (α-Pro), 66.0 (OCH_2CH=CH_2, Aloc), 67.6 (OCH_2CH=CH_2, ester), 72.2 (α-hArg), 78.8 (OC(CH_3)$_3$), 82.8 (OC(CH_3)$_3$), 117.3 (OCH_2CH=CH_2, Aloc), 118.4 (OCH_2CH=CH_2, ester), 121.3 (α-vAla), 126.7 (aryl-4 Phe), 128.3 (aryl-2 Phe), 129.1 (aryl-3 Phe), 131.3 (OCH_2-CH=CH_2, ester), 132.4 (OCH_2CH=CH_2, Aloc), 136.6 (aryl-1 Phe), 144.4 (β-vAla), 152.9 (C(O)O, Boc), 154.4 and 155.3 (C(O)O, Aloc rotam), 155.8 (C=N), 163.2 (C(O)O, Boc), 166.1 (C(O)NH, Phe-Dpr), 170.4 (C(O)NH), 170.4 (C(O)CH_3), 170.8 (C(O)OAll), 171.9 (C(O)NH) and 172.6 (C(O)NH).

Allyl 2(*S*)-phenylacetylamino-3-[[4(*S*)-[[2(*R*)-[[2(*R,S*)-hydroxy-3(*S*)-[[[1-(allyloxycarbonyl)pyrrolidin-2(*S*)-yl]carbonyl]amino]-6-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]methyl]amino]hexanoyl]amino]-3-phenylpropanoyl]amino]pent-2(*E*)-enoyl]amino]propanoate (131b)

Prepared from tripeptide **97** (989 mg, 1.35 mmol), dipeptide **130b** (594 mg, 1.50 mmol), DiPEA (522 μL, 3.00 mmol) and TBTU (485 mg, 1.50 mmol) according to the same procedure as described for the synthesis of **110** [Chapter Five]. After aqueous work-up, the product was purified by preparative TLC (CH_2Cl_2/THF/EtOH, 120:80:1.5), to give **131b** as a white foam (1.25 g, 85.8%).

^{1}H-NMR (DMSO-d_6): 1.01 (d, *J*=6.7, 3H, vAla δ-H), 1.07-1.55 (m, 4H, hArg γ- and δ-H), 1.39 (s, 9H, Boc), 1.48 (s, 9H, Boc), 1.62-1.89 (m, 3H, Pro β- and γ-H), 2.01 (m, 1H, Pro β-H), 2.90 (bd, *J*=6.0, 2H, Phe β-H), 3.21 (m, 2H, hArg ε-H), 3.30-3.67 (m, 4H, Pro δ-H, Dpr β-H), 3.48 (s (overlapping), 2H, Pac CH_2Ph), 3.87 (m, 1H, hArg α-H), 3.99 (m, 1H, hArg β-H), 4.21 (m, 1H, Pro α-H), 4.30-4.62 (m, 7H, Phe α-H, vAla γ-H, Dpr α-H and 2x OCH_2CH=CH_2), 5.02-5.38 (m, 4H, 2x OCH_2CH=CH_2), 5.69-5.98 (m, 4H, hArg OH, vAla α-H and 2x OCH_2CH=CH_2), 6.53 (dd, *J*=5.4 and 15.3, 1H, vAla β-H), 7.12-7.35 (m, 10H, Phe aryl, Pac aryl), 7.52-7.78 (m, 2H, hArg β-NH, Phe α-NH), 8.11-8.30 (m, 3H, hArg ε-NH, vAla γ-NH, Dpr β-NH), 8.52 (d, *J*=7.0, 1H, Dpr α-NH) and 11.52 (bs, 1H, hArg ω-NH).

***N*-Ethyl morpholinium 2(*S*)-acetylamino-3-[[4(*S*)-[[2(*R*)-[[2(*R,S*)-hydroxy-3(*S*)-[[(pyrrolidin-2(*S*)-yl)carbonyl]amino]-6-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]methyl]-amino]hexanoyl]amino]-3-phenylpropanoyl]amino]pent-2(*E*)-enoyl]amino]propanoate (132a)**

Prepared from **131a** (260 mg, 0.260 mmol) according to the same procedure as described for the synthesis of **111** [Chapter Five]. The product was purified by preparative TLC (CH_2Cl_2/MeOH/NEM, 85:20:10), to give **132a** as a white powder (195 mg, 75.8%).

^{1}H-NMR (DMSO-d_6): 0.98 (t, *J*=6.7, 3H, *N*-$CH_2C\underline{H}_3$ NEM), 1.01 (t, *J*=6.7, 3H, vAla δ-H), 1.11-2.01 (m, 8H, Pro β- and γ-H, hArg γ- and δ-H), 1.38 (s, 9H, Boc), 1.47 (s, 9H, Boc), 1.81 (s, 3H, Ac), 2.23-2.48 (m, 6H, *N*-CH_2 NEM), 2.73-3.02 (m, 4H, Phe β-H), 3.10-3.63 (m, 8H, hArg ε-H, Dpr β-H, *O*-CH_2 NEM), 3.68 (m, 1H, Pro α-H), 3.71-4.12 (m, 3H, hArg α- and β-H, Dpr α-H), 4.28-4.61 (m, 2H, Phe α-H, vAla γ-H), 5.90 (d *J*=15.2, 1H, vAla α-H), 6.09 (bs, <1H, hArg OH), 6.50 (dd, *J*=15.2 and 4.7, 1H, vAla β-H), 7.10-7.32 (m, 5H, Phe aryl), 7.55 8.31 (m, 5H, NH) and 11.47 (bs, 1H, hArg ω-NH).

***N*-Ethyl morpholinium 2(*S*)-phenylacetylamino-3-[[4(*S*)-[[2(*R*)-[[2(*R,S*)-hydroxy-3(*S*)-[[(pyrrolidin-2(*S*)-yl)carbonyl]amino]-6-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]-methyl]amino]hexanoyl]amino]-3-phenylpropanoyl]amino]pent-2(*E*)-enoyl]amino]propanoate (132b)**

Prepared from **131b** (1.30 g, 1.21 mmol) according to the same procedure as described for the synthesis of **111** [Chapter Five]. The product precipitated from CH_2Cl_2/MeOH/NEM (120:80:1.5), and was collected by centrifugation, to give **132b** as an off-white powder (0.90 g, 78.2%).
^{1}H-NMR (DMSO-d_6): 0.99 (t, *J*=6.7, 3H, *N*-$CH_2\underline{C}H_3$ NEM), 1.01 (t, *J*=6.7, 3H, vAla δ-H), 1.11-1.53 (m, 4H, hArg γ- and δ-H), 1.40 (s, 9H, Boc), 1.48 (s, 9H, Boc), 1.56-1.73 (m, 3H, Pro β- and γ-H), 1.97 (m, 1H, Pro β-H), 2.21-2.45 (m, 6H, *N*-$C\underline{H}_2$ NEM), 2.75-3.00 (m, 4H, Pro δ-H, Phe β-H), 3.11-3.63 (m, 6H, hArg ε-H, Dpr β-H, *O*-CH_2 NEM), 3.46 (bs overlapping, 2H, CH_2 Pac), 3.69 (m, 1H, Pro α-H), 3.90-4.12 (m, 3H, hArg α- and β-H, Dpr α-H), 4.30-4.61 (m, 2H, Phe α-H, vAla γ-H), 5.90 (d *J*=15.2, 1H, vAla α-H), 6.08 (bs, 1H, hArg OH), 6.50 (dd, *J*=15.2 and 4.7, 1H, vAla β-H), 7.10-7.38 (m, 10H, aryl), 7.72 (bd, *J*=8.2, 1H, NH), 7.90-8.02 (m, 2H, NH), 8.07-8.20 (m, 2H, NH), 8.28 (bt, *J*=6.0, 1H, hArg ε-NH) and 11.50 (bs, 1H, hArg ω-NH).

(2*S*,5*S*,11*S*,14*R*,17(*R*,*S*),18*S*)-*N*-[11-Methyl-14-benzyl-17-hydroxy-18-[[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]methyl]amino]propyl]-4,8,13,16,20-pentaoxo-2,3,4,5,6,7,8,11,12,13,14,15,16,17,18,19,20,20a-octadecahydro-1*H*-3a,7,12,15,19-pentaaza-cyclopentacyclononadecen-5-yl]acetamide (133a)

Prepared from **132a** (189 mg, 0.191 mmol) according to the same procedure as described for the synthesis of **112** [Chapter Five]. The product was purified by preparative TLC (CH_2Cl_2/THF/EtOH, 60:40:1), to give **133a** as a white foam (24.5 mg, 15.0%).
^{1}H-NMR ($CDCl_3$, 400.1 MHz): 0.89 (d, *J*=6.7, 3H, vAla δ-H), 1.47 (s, 9H, Boc), 1.52 (s, 9H, Boc), 1.48-1.68 (m, 4H, hArg γ- and δ-H), 1.68-2.15 (m, 3H, Pro β- and γ-H), 1.97 (s, 3H, Ac), 2.23 (m, 1H, Pro β-H), 2.71-2.90 (m, 2H, Phe β-H, Dpr β-H), 3.18 (dd, *J*=13.1 and 4.7, 1H, Phe β-H), 3.31 (m, 1H, hArg ε-H), 3.58 (m, 1H, Pro δ-H) 3.61-3.85 (m, 3H, Pro δ-H, hArg ε-H and OH), 4.07 (m, 1H, hArg α-H), 4.21-4.55 (m, 5H, Pro α-H, hArg β-H, Phe α-H, vAla γ-H, Dpr β-H), 4.79-4.98 (m, 2H, Dpr α-H, vAla γ-NH), 5.98 (dd, *J*=15.3 and 1.9, 1H, vAla α-H), 6.35 (bd, *J*=7.5, 1H, Dpr α-NH), 6.62 (dd, *J*=15.3 and 2.4, 1H, vAla β-H), 6.93 (d, *J*=7.6, 1H, Phe α-NH), 7.19-7.39 (m, 5H, Phe aryl), 7.61 (bd, *J*=10.0, 1H, hArg β-NH), 7.91 (bd, *J*=10.2, 1H, Dpr β-NH), 8.47 (bt, *J*=6.3, 1H, hArg ε-NH) and 11.54 (bs, 1H, hArg ω-NH). ^{13}C-NMR ($CDCl_3$): 19.2 (δ-vAla), 22.9 (C(O)$\underline{C}H_3$), 25.0 (γ-Pro), 26.6 (δ-hArg), 27.3 (γ-hArg), 28.1 (C(O)OC($\underline{C}H_3)_3$), 29.1 (C(O)OC($\underline{C}H_3)_3$), 30.8 (β-Pro), 39.9 (ε-hArg), 40.4 (β-Phe), 40.9 (β-Dpr), 45.6 (γ-vAla), 48.2 (δ-Pro), 49.3 (α-Dpr), 53.2 (β-hArg), 55.8 (α-Phe), 60.6 (α-Pro), 72.6 (α-hArg), 79.7 (C(O)O$\underline{C}(CH_3)_3$), 83.2 (C(O)O$\underline{C}(CH_3)_3$), 123.4 (α-vAla), 127.3 (aryl-4 Phe), 128.8 (aryl-2 Phe), 129.3 (aryl-3 Phe), 136.4 (aryl-1 Phe), 142.4 (β-vAla), 153.3 (C(O)O, Boc), 156.9 (C=N), 163.4 (C(O)O, Boc), 165.5 (C(O)NH, vAla-Dpr), 169.2 (C(O)NH), 169.8 ($\underline{C}(O)CH_3$), 170.1 (C(O)NH), 171.1 (C(O)NH) and 172.7 (C(O)NH).

(2*S*,5*S*,11*S*,14*R*,17(*R*,*S*),18*S*)-*N*-[11-Methyl-14-benzyl-17-hydroxy-18-[[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]methyl]amino]propyl]-4,8,13,16,20-pentaoxo-2,3,4,5,6,7,8,11,12,13,14,15,16,17,18,19,20,20a-octadecahydro-1*H*-3a,7,12,15,19-pentaaza-cyclopentacyclononadecen-5-yl]phenylacetamide (133b)

Prepared from **132b** (605 mg, 0.637 mmol) according to the same procedure as described for the synthesis of **112** [Chapter Five]. The product was purified by preparative TLC (CH_2Cl_2/THF/EtOH, 120:80:1.5), to give **133b** as a white foam (346 mg, 58.2%).
^{1}H-NMR (DMSO-d_6, 400.1 MHz): 1.00 (d, *J*=6.8, 3H, vAla δ-H), 1.41 (s, 9H, Boc), 1.50 (s, 9H, Boc), 1.38-1.61 (m, 4H, hArg γ- and δ-H), 1.73 (m, 2H, Pro γ-H), 1.88 (m, 1H, Pro β-H), 2.05 (m, 1H, Pro

β-H), 2.61-2.83 (m, 3H, Phe β-H, Dpr β-H), 3.20-3.38 (m, 3H, hArg ε-H, Pro δ-H), 3.42 (bs, 2H, Pac C$\underline{H}_2$Ph), 3.53 (m, 1H, Pro δ-H), 4.00 (m, 1H, Dpr β-H), 4.04 (m, 1H, hArg α-H), 4.08-4.20 (m, 2H, hArg β-H and OH), 4.32 (m, 1H, vAla γ-H), 4.37-4.51 (m, 2H, Pro α-H, Dpr α-H), 4.60 (m, 1H, Phe α-H), 5.95 (dd *J*=15.3 and 2.6, 1H, vAla α-H), 6.50 (dd, *J*=15.3 and 2.8, 1H, vAla β-H), 7.08-7.30 (m, 10H, aryl), 7.97 (d, *J*=9.2, 1H, hArg β-NH), 8.18 (d, *J*=7.7, 1H, vAla γ-NH), 8.25-8.39 (m, 2H, hArg ε-NH, Phe α-NH), 8.45 (d, *J*=7.0, 1H, Dpr α-NH), 8.59 (bt, *J*=10.6, 1H, Dpr β-NH) and 11.54 (bs, 1H, hArg ω-NH).^{13}C-NMR ($CDCl_3$): 19.2 (δ-vAla), 24.9 (γ-Pro), 26.7 (δ-hArg), 27.1 (γ-hArg), 28.0 (C(O)OC(C$\underline{H}_3$)$_3$), 28.5 (C(O)OC(C$\underline{H}_3$)$_3$), 30.8 (β-Pro), 39.9 (ε-hArg), 40.3 (β-Phe), 40.5 (β-Dpr), 43.0 (C$\underline{H}_2$Ph, Pac), 45.5 (γ-vAla), 48.2 (δ-Pro), 49.4 (α-Dpr), 53.1 (β-hArg), 55.7 (α-Phe), 60.5 (α-Pro), 72.5 (α-hArg), 79.5 (C(O)OC(CH$_3$)$_3$), 83.1 (C(O)OC(CH$_3$)$_3$), 123.2 (α-vAla), 127.1 (aryl-4 Pac), 127.2 (aryl-4 Phe), 128.7 (aryl-2 Phe, aryl-2 Pac), 129.2 (aryl-3 Phe, aryl-3 Pac), 134. 4 (aryl-1 Pac), 136.4 (aryl-1 Phe), 142.7 (β-vAla), 153.2 (C(O)O, Boc), 156.9 (C=N), 163.4 (C(O)O, Boc), 165.4 (C(O)NH, vAla-Dpr), 169.2 (C(O)NH), 170.0 (C(O)NH), 170.7 (C(O)NH), 171.1 (C(O)NH) and 172.7 (C(O)NH).

(2*S*,5*S*,11*S*,14*R*,18*S*)-*N*-[11-Methyl-14-benzyl-18-[[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]methyl]amino]propyl]-4,8,13,16,17,20-hexaoxo-2,3,4,5,6,7,8,11,12,13,14,15,16,17,18,19,20,20a-octadecahydro-1*H*-3a,7,12,15,19-pentaaza-cyclopentacyclononadecen-5-yl]acetamide (134a)

Prepared from **133a** (25.6 mg, 29.9 μmol) according to the same procedure as described for the synthesis of **116** [Chapter Five], to give **134a** as an off-white foam (22.1 mg, max 87%). No NMR spectra were recorded due to the small amount of material available.

(2*S*,5*S*,11*S*,14*R*,18*S*)-*N*-[11-Methyl-14-benzyl-18-[[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]methyl]amino]propyl]-4,8,13,16,17,20-hexaoxo-2,3,4,5,6,7,8,11,12,13,14,15,16,17,18,19,20,20a-octadecahydro-1*H*-3a,7,12,15,19-pentaaza-cyclopentacyclononadecen-5-yl]phenylacetamide (134b)

Prepared from **133b** (221 mg, 0.237 mmol) according to the same procedure as described for the synthesis of **116** [Chapter Five], to give **134b** as a glass (214 mg, *ca* 70%, based upon the ^{1}H-NMR-spectrum, which showed the presence of *ca* 20% starting material).

^{1}H-NMR (DMSO-d_6): 1.04 (d, *J*=6.7, 3H, vAla δ-H), 1.41 (s, 9H, Boc), 1.50 (s, 9H, Boc), 1.31-1.63 (m, 4H, hArg γ- and δ-H), 1.68-1.90 (m, 3H, Pro β- and γ-H), 2.10 (m, 1H, Pro β-H), 2.68-3.00 (m, 3H, Phe β-H, Dpr β-H), 3.10-3.35 (m, 3H, hArg ε-H, Pro δ-H), 3.43 (bs, 2H, Pac C$\underline{H}_2$Ph), 3.52 (m, 1H, Pro δ-H), 3.98 (m, 1H, Dpr β-H), 4.30-4.58 and 4.61 (m, 5H, Pro α-H, hArg α-H, Phe α-H, vAla γ-H, Dpr α-H), 5.70 (dd *J*=14.9 and 2.3, 1H, vAla α-H), 6.59 (dd, *J*=14.9 and 2.3, 1H, vAla β-H), 7.08-7.35 (m, 10H, aryl), 8.00-8.55 (m, 5H, NH), 8.18 (d, *J*=7.7, 1H, vAla γ-NH), 8.25-8.39 (m, 2H, hArg ε-NH, Phe α-NH), 8.81 (d, *J*=5.7, 1H, NH) and 11.55 (bs, 1H, hArg ω-NH).

(2*S*,5*S*,11*S*,14*R*,18*S*)-*N*-[11-Methyl-14-benzyl-18-[[[imino(amino)methyl]amino]propyl]-4,8,13,16,17,20-hexaoxo-2,3,4,5,6,7,8,11,12,13,14,15,16,17,18,19,20,20a-octadecahydro-1*H*-3a,7,12,15,19-pentaaza-cyclopentacyclononadecen-5-yl]acetamide (117a)

Prepared from **134a** (21.5 mg, max 25.2 μmol) according to the same procedure as described for the synthesis of **1b** [Chapter Five]. The crude product was purified by reverse phase HPLC; column: Waters delta-Pak C_{18} 40x100 mm (Waters 37688); mobile phase: (MeCN/H_2O, 3:2)/H_2O/phosphate buffer (0.5 M, pH= 2.1), gradient from 20:60:20 to 80:0:20 in 57 min; flow: 40 mL/min. The product

was desalted on the same column ($MeCN/H_2O$, 3:2)/H_2O/HCl_{aq} (0.1 N), 0:80:20 15 min, then 80:18:2), and lyophilized to give **117a**·1.9HCl·$20H_2O$ (based on peptide content) as a fluffy white powder (12.9 mg, 41.2% from **134a**); analytical HPLC, ≥98.6%.

^{1}H-NMR (D_2O, 400.1 MHz): 0.90 (d, *J*=7.3, 1H, vAla δ-H), 1.45-1.58 (m, 2H, kArg γ- and δ-H), 1.64 (m, 1H, kArg δ-H), 1.73-1.86 (m, 2H, Pro β- and γ-H), 1.86-1.99 (m, 2H, Pro γ-H, kArg γ-H), 1.94 (s, 3H, Ac), 2.26 (m, 1H, Pro β-H), 2.82-2.92 (m, 2H, Phe β-H, Dpr β-H), 3.03 (dd, *J*=13.2 and 5.8, 1H, Phe β-H), 3.16 (m, 2H, kArg ε-H), 3.39 (m, 1H, Pro δ-H), 3.70 (m, 1H, Pro δ-H), 4.04 (dd, *J*=10.9 and 2.4, 1H, kArg β-H), 4.14 (dd, *J*=12.8 and 5.9, 1H, Dpr β-H), 4.26 (m, 1H, vAla γ-H), 4.46 (m, 1H, Pro α-H), 4.51 (dd, *J*=9.2 and 5.9, 1H, Phe α-H), 4.55 (m, *J*=11.2 and 6.0, 1H, Dpr α-H), 5.80 (dd, *J*=15.6 and 2.2, 1H, vAla α-H), 6.61 (dd, *J*=15.6 and 2.6, 1H, vAla β-H), 7.19-7.22 (m, 2H, Phe aryl) and 7.25-7.36 (m, 3H, Phe aryl). FAB-MS *m/z* 654 (MH^+) and 672 ($MH^+ + H_2O$).

(2*S*,5*S*,11*S*,14*R*,18*S*)-*N*-[11-Methyl-14-benzyl-18-[[[imino(amino)methyl]amino]propyl]-4,8,13,16,17,20-hexaoxo-2,3,4,5,6,7,8,11,12,13,14,15,16,17,18,19,20,20a-octadecahydro-1*H*-3a,7,12,15,19-pentaaza-cyclopentacyclononadecen-5-yl]phenylacetamide (117b)

Prepared from **134b** (149 mg, *ca* 0.160 mmol) according to the same procedure as described for the synthesis of **1b** [Chapter Five]. The crude product was purified by reverse phase HPLC; column: Waters delta-Pak C_{18} 40x100 mm (Water 37688); mobile phase: ($MeCN/H_2O$, 3:2)/H_2O/phosphate buffer (0.5 M, pH= 2.1), gradient from 20:60:20 to 80:0:20 in 57 min; flow: 20 mL/min. The product was desalted on the same column ($MeCN/H_2O$, 3:2)/H_2O/HCl_{aq} (0.1 N), 0:80:20 15 min, then 80:18:2), and lyophilized to give **117b**·1.3HCl·$16H_2O$ (based on peptide content) as a fluffy white powder (66.9 mg, 39.2 %); analytical HPLC, ≥98.3%.

^{1}H-NMR (D_2O, 400.1 MHz): 0.91 (d, *J*=7.3, 1H, vAla δ-H), 1.48-1.59 (m, 2H, kArg γ- and δ-H), 1.65 (m, 1H, kArg δ-H), 1.71-1.86 (m, 2H, Pro β- and γ-H), 1.86-2.00 (m, 2H, Pro γ-H, kArg γ-H), 2.24 (m, 1H, Pro β-H), 2.86-2.94 (m, 2H, Phe β-H, Dpr β-H), 3.04 (dd, *J*=13.1 and 5.8, 1H, Phe β-H), 3.17 (m, 2H, kArg ε-H), 3.38 (m, 1H, Pro δ-H), 3.58 (s, 2H, Pac CH_2), 3.68 (m, 1H, Pro δ-H), 4.05 (dd, *J*=10.9 and 2.4, 1H, kArg β-H), 4.17 (dd, *J*=12.8 and 5.9, 1H, Dpr β-H), 4.27 (m, 1H, vAla γ-H), 4.45 (m, 1H, Pro α-H), 4.52 (dd, *J*=9.2 and 5.9, 1H, Phe α-H), 4.56 (m, *J*=11.3 and 6.0, 1H, Dpr α-H), 5.81 (dd, *J*=15.6 and 2.2, 1H, vAla α-H), 6.62 (dd, *J*=15.6 and 2.6, 1H, vAla β-H), 7.20-7.38 (m, 10H, Phe aryl, Pac aryl). ^{13}C-NMR ($CDCl_3$): 19.5 (δ-vAla), 26.0 (δ-kArg), 27.2 (γ-kArg), 27.4 (γ-Pro), 33.0 (β-Pro), 41.7 (β-Dpr), 42.4 (β-Phe), 43.4 (ε-hArg), 44.2 (CH_2 Pac), 48.7 (γ-vAla), 51.4 (δ-Pro), 53.3 (α-Dpr), 57.2 (β-kArg), 57.6 (α-Phe), 63.3 (α-Pro), 97.2 (α-kArg), 124.0 (α-vTyr), 130.0 (aryl-4 Pac), 130.0 (aryl-4 Phe), 131.5 (aryl), 131.6 (aryl), 131.8 (aryl), 132.1 (aryl), 137.4 (aryl-1), 138.5 (aryl-1), 147.9 (β-vTyr), 159.4 (C=N), 170.5 (C(O)NH), 173.3 (C(O)NH), 173.7 (C(O)NH), 174.4 (C(O)NH), 176.1 (C(O)NH) and 177.2 (C(O)NH). FAB-MS *m/z* 730 (MH^+) and 748 ($MH^+ + H_2O$).

Allyl 2(*R*)-amino-3-phenylpropanoate hydrochloride (137)

A stirred suspension of D-phenylalanine (4.98 g, 30.1 mmol) and *p*-TsOH·H_2O (8.99 g, 47.3 mmol) in allyl alcohol/benzene (45 mL, 4:5) was refluxed in a reaction flask equipped with a Dean-Stark trap and a drying tube ($CaCl_2$). After 5 h, the clear reaction mixture was allowed to cool to room temperature. Et_2O (50 mL) was added, and the resulting precipitate was collected by vacuum filtration. The precipitate was partitioned between Et_2O and saturated aqueous $NaHCO_3$. The layers were separated, and the basic aqueous layer was extracted with Et_2O (2x). The combined organic layers were washed with brine, dried (Na_2SO_4), filtrated, and concentrated *in vacuo*, to give an oily residue

(5.51 g). This residue was treated with 4 N HCl in Et_2O at 0 °C, to give, after filtration and drying *in vacuo*, **137** as a white powder (5.12 g, 78.7%); 164-165.5 °C

^{1}H-NMR (DMSO-d_6): 3.17 (m, 2H, β-H), 4.25 (m, 1H, α-H), 4.57 (d, *J*=5.3, 2H, $OC\underline{H}_2CH{=}CH_2$), 5.20 (m, 2H, $OCH_2CH{=}C\underline{H}_2$), 5.73 (m, 1H, $OCH_2C\underline{H}{=}CH_2$), 7.12-7.39 (m, 5H, aryl) and 8.77 (bs, 3H, NH_3^+).

Allyl 2(*R*)-[[4(*S*)-[(*tert*-butyloxycarbonyl)amino]-5-[4-(*tert*-butyloxy)phenyl]pent-2-(*E*)-enoyl]amino]-3-phenylpropanoate (138)

Prepared from **100** (1.47 g, 3.65 mmol), **137** (0.89 g, 4.12 mmol), DiPEA (1.40 mL, 8.04 mmol), and TBTU (1.19 g, 3.70 mmol) according to the same procedure as described for the synthesis of **107** [Chapter Four], to give **138** as as colourless crystals (1.85 g, 92.0%); 129-.131.5 °C

^{1}H-NMR ($CDCl_3$): 1.30 (s, 9H, *t*-Bu), 1.38 (s, 9H, Boc), 2.80 (m, 2H, vTyr δ-H), 3.14 (bd, *J*=5.3, 2H, Phe β-H), 4.35-4.58 (m, 2H, vTyr γ-H and γ-NH), 4.60 (bd, *J*=5.8, 2H, $OC\underline{H}_2CH{=}CH_2$), 4.96 (m, 1H, Phe α-H), 5.27 (m, 2H, $OCH_2CH{=}C\underline{H}_2$), 5.76 (dd, *J*=15.2 and 1.5, 1H, vTyr α-H), 5.78-5.99 (m, 1H, Phe α-NH, $OCH_2C\underline{H}{=}CH_2$), 6.80 (dd, *J*=15.2 and 4.7, 1H, vTyr β-H), 6.90 and 7.08 (AB-system, *J*=8.2, 4H, vTyr aryl), 6.95-7.10 (m overlapping, 2H, Phe aryl) and 7.18-7.30 (m, 3H, aryl). ^{13}C-NMR ($CDCl_3$): 28.3 ($OC(\underline{C}H_3)_3$), 28.8 ($C(O)OC(\underline{C}H_3)_3$), 37.8 (δ-vTyr), 40.4 (β-Phe), 52.4 (γ-vTyr), 53.1 (α-Phe), 66.1 ($O\underline{C}H_2CH{=}CH_2$), 78.3 ($O\underline{C}(CH_3)_3$), 79.8 ($C(O)O\underline{C}(CH_3)_3$), 119.1 ($OCH_2CH{=}\underline{C}H_2$), 122.7 (α-vTyr), 124.0 (aryl-3 vTyr), 127.1 (aryl-4 Phe), 128.5 (aryl-2 Phe), 129.3 (aryl-3 Phe), 129.8 (aryl-2 vTyr), 131.3 ($OCH_2\underline{C}H{=}CH_2$), 131.3 (aryl-1 vTyr), 135.6 (aryl-1 Phe), 144.4 (β-vTyr), 154.1 (aryl-4 vTyr), 155.0 (C(O)O, Boc), 165.5 (C(O)NH) and 171.0 (C(O)OAll). FAB-HRMS: calcd for $[C_{32}H_{42}N_2O_6 + H]^+$ 551.3121, found 551.3100.

Allyl 2(*R*)-[[4(*S*)-amino-5-[4-(*tert*-butyloxy)phenyl]pent-2-(*E*)-enoyl]amino]-3-phenylpropanoate (139)

Prepared from **138** (1.27 g, 2.31 mmol) according to the same procedure as described for the synthesis of **109** [Chapter Four], to give **139** as a glass (1.23 g, 100%) contaminated with *ca* 1 equiv of 2,6-lutidine.

^{1}H-NMR ($CDCl_3$): 1.30 (s, 9H, *t*-Bu), 2.80-3.14 (m, 4H, vTyr δ-H, Phe β-H), 4.00 (m, 1H, vTyr γ-H), 4.52 (bd, *J*=5.3, 2H, $OC\underline{H}_2CH{=}CH_2$), 4.74 (m, 1H, Phe α-H), 5.21 (m, 2H, $OCH_2CH{=}C\underline{H}_2$), 5.68-5.90 (m, 1H, $OCH_2C\underline{H}{=}CH_2$), 6.04 (bd, *J*=15.0, 1H, vTyr α-H), 6.63 (dd, *J*=15.2 and 7.1, 1H, vTyr β-H), 6.90 and 7.06 (AB-system, *J*=8.1, 4H, vTyr aryl) and 7.00-7.28 (m overlapping, 5H, Phe aryl).

Allyl 2(*R*)-[[4(*S*)-[[2(*R*)-[[2-(*R,S*)-hydroxy-3(*S*)-[[[1-(allyloxycarbonyl)pyrrolidin-2(*S*)-yl]-carbonyl]amino]-6-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]methyl]amino]-hexanoyl]amino]-3-phenylpropanoyl]amino]-5-[4-(*tert*-butyloxy)phenyl]pent-2(*E*)-enoyl]amino]-3-phenylpropanoate (140)

Prepared from tripeptide **97** (1.50 g, 2.05 mmol), dipeptide **139** (1.23 g, 2.31 mmol + 2,6-lutidine, *vide supra*), DiPEA (0.40 mL, 2.30 mmol) and TBTU (0.74 g, 2.30 mmol) according to the same procedure as described for the synthesis of **110** [Chapter Five]. After aqueous work-up, the product was purified by centrifugal chromatography (CH_2Cl_2/EtOAc/EtOH, 95:3:2), to give **140** as a white foam (1.39 g, 58.2%).

^{1}H-NMR (DMSO-d_6, 400.1 MHz): 1.07-1.50 (m, 4H, hArg γ- and δ-H), 1.20 (s, 9H, *t*-Bu), 1.41 (s, 9H, Boc), 1.47 (s, 9H, Boc), 1.68-1.83 (m, 3H, Pro β- and γ-H), 1.97 (m, 1H, Pro β-H), 2.50-2.73 (m, 3H, Phe β-H, vTyr δ-H), 2.80 (m, 1H, Phe β-H), 3.19 (m, 2H, hArg ε-H), 3.33 (m, 2H, Pro δ-H), 3.81 (m,

1H, hArg α-H), 3.91 (m, 1H, hArg β-H), 4.18 (m, 1H, Pro α-H), 4.32-4.60 (m, 7H, Phe α-H, vTyr γ-H, Phe α-H and 2x $OC\underline{H}_2CH=CH_2$), 5.03-5.30 (m, 4H, 2x $OCH_2CH=C\underline{H}_2$), 5.70-5.93 (m, 3H, hArg OH, 2x $OCH_2C\underline{H}=CH_2$), 5.95 (d, *J*=15.1, 1H, vTyr α-H), 6.58 (dd, *J*=15.2 and 5.3, 1H, vTyr β-H), 6.87 (part of AB-system, *J*=8.4, vTyr aryl), 6.89-6.97 (m, 2H, Phe aryl), 7.08-7.32 (m, 11H, Phe aryl, vTyr aryl), 7.45-7.60 (m, 2H, NH), 8.20 (m, 1H, NH), 8.32 (m, 1H, NH), 8.57 (m, 1H, NH) and 11.50 (bs, 1H, hArg ω-NH). ^{13}C-NMR (DMSO-d_6, 100 MHz): 22.5 and 23.4 (γ-Pro rotam), 24.7 (δ-hArg), 25.0 (γ-hArg), 27.0 ($C(O)OC(\underline{C}H_3)_3$), 27.6 ($C(O)OC(\underline{C}H_3)_3$), 28.0 ($OC(\underline{C}H_3)_3$), 29.3 and 30.7 (β-Pro rotam), 36.0 (β-Phe), 38.0 (δ-vTyr), 38.2 (β-Phe), 39.8 (ε-hArg), 45.9 and 46.5 (δ-Pro rotam), 50.6 and 50.7 (β-hArg rotam), 50.8 (γ-vTyr), 52.5 (α-Phe), 53.2 (α-Phe), 58.7 and 59.3 (α-Pro rotam), 64.2 and 64.4 ($O\underline{C}H_2CH=CH_2$, Aloc rotam), 64.3 ($O\underline{C}H_2CH=CH_2$, ester), 71.3 (α-hArg), 77.1 ($O\underline{C}(CH_3)_3$), 77.6 ($C(O)O\underline{C}(CH_3)_3$), 82.4 ($C(O)O\underline{C}(CH_3)_3$), 115.7 and 116.3 ($OCH_2CH=\underline{C}H_2$, Aloc rotam), 117.1 ($OCH_2CH=\underline{C}H_2$, ester), 122.6 (α-vTyr), 122.9 (aryl-2 vTyr), 125.8 (aryl-4 Phe), 126.0 (aryl-4 Phe), 127.4 (aryl-2 Phe), 127.7 (aryl-2 Phe), 128.7 (aryl-3 Phe), 129.3 (aryl-3 Phe), 131.7 ($OCH_2\underline{C}H=CH_2$, ester), 132.0 (aryl-1 vTyr), 132.7 and 132.9 ($OCH_2\underline{C}H=CH_2$, Aloc rotam), 136.5 (aryl-1 Phe), 136.6 (aryl-1 Phe), 142.3 (β-vTyr), 151.3 (C(O)O, Boc), 153.0 (aryl-4 vTyr), 153.1 and 153.3 (C(O)O, Aloc rotam), 154.6 (C=N), 162.6 (C(O)O, Boc), 164.1 (C(O)NH), 169.3 (C(O)NH), 170.7 and 170.8 (C(O)NH and C(O)OAll), 171.0 and 171.2 (C(O)NH, Pro-hArg rotam).

***N*-Ethyl morpholinium 2(*R*)-[[4(*S*)-[[2(*R*)-[[2-(*R,S*)-hydroxy-3(*S*)-[[(pyrrolidine)2(*S*)-yl)carbonyl]amino]-6-[[(*tert*-butyloxycarbonyl)imino[(*tert*-butyloxycarbonyl)amino]methyl]amino]-hexanoyl]amino]-3-phenylpropanoyl]amino]-5-[4-(*tert*-butyloxy)phenyl]pent-2(*E*)-enoyl]amino]-3-phenylpropanoic acid (141)**

Prepared from tripeptide **140** (639 mg, 0.548 mmol) according to the same procedure as described for the synthesis of **112** [Chapter Five]. The volatiles were removed *in vacuo* and the residue was dissolved in THF/NEM (97:3, *ca* 7 mL). Within a few minutes a precipitate formed, which was collected by centrifugation, to give **132b** as a white powder (470 mg, 74.2%); analytical HPLC, ≥95%.

^{1}H-NMR (DMSO-d_6): 0.95 (t, *J*=6.7, 3H, *N*-$CH_2C\underline{H}_3$ NEM), 1.23 (s, 9H, *t*-Bu), 1.30-1.83 (m, 7H, Pro β- and γ-H, hArg γ- and δ-H), 1.38 (s, 9H, Boc), 1.47 (s, 9H, Boc), 1.93 (m, 1H, Pro β-H), 2.22-2.37 (m, 6H, *N*-$C\underline{H}_2CH_3$ NEM), 2.50-3.70 (m, 15H, Pro δ-H, hArg ε-H, Phe α-H and β-H (2x), vTyr δ-H, *O*-CH_2 NEM), 3.91 (m, 1H, hArg α-H), 3.97 (m, 1H, hArg β-H), 4.26-4.60 (m, 3H, Pro α-H, Phe α-H, vTyr γ-H), 5.90 (bs overlapping, 1H, hArg OH), 5.95 (bd, *J*=15.2, 1H, vTyr α-H), 6.50 (dd, *J*=15.2 and 4.7, 1H, vTyr β-H), 6.8-6.97 (m, 4H, aryl), 7.05-7.38 (m, 11H, aryl), 7.61 (m, 1H, NH), 7.98 (d, *J*=9.3, 1H, NH), 8.14 (m, *J=8.2, 1H*, NH), 8.18-8.32 (m, 2H, NH) and 11.48 (bs, 1H, hArg ω-NH).

(2*S*, 5*R*, 10*S*, 13*R*, 16(*R,S*), 17*S*)-*N*-[3-[5,13-Dibenzyl-10-[4-(*tert*-butyloxy)benzyl]-16-hydroxy-4, 7, 12, 15, 19-pentaoxo-1, 2, 3, 4, 5, 6, 7, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 19a-octadecahydro-1*H*-3a, 6, 11, 14, 18-pentaaza-cyclopentacyclooctadecen-17-yl]propylamino[(*tert*-butyloxycarbonylimino)methyl]]*tert*-butyloxycarbonylamine (142)

Prepared from **141** (412 mg, 0.356 mmol) according to the same procedure as described for the synthesis of **112** [Chapter Five]. The product was purified by preparative TLC (CH_2Cl_2/THF, 3:1), to give **142** as a white foam (254 mg, 69.2%).

^{1}H-NMR (DMSO-d_6, 400.1 MHz): 1.23 (s, 9H, *t*-Bu), 1.26-1.60 (m, 4H, hArg γ- and δ-H), 1.38 (s, 9H, Boc), 1.48 (s, 9H, Boc), 1.60-1.78 (m, 3H, Pro β and γ-H), 1.95 (m, 1H, Pro β-H), 2.50-2.92 (m, 5H, Phe β-H (2x), vTyr δ-H), 2.92-3.10 (m, 3H, hArg ε-H, Phe β-H), 3.35 (m, 1H, Pro δ-H), 3.49 (m, 1H,

Pro δ-H), 3.75 (m, 1H, hArg β-H), 3.91 (m, 1H, hArg α-H), 4.09 (m, 1H, Pro α-H), 4.28 (m, 1H, Phe α-H), 4.52 (m, 1H, vTyr γ-H), 4.78 (m, 1H, Phe α-H), 6.03 (m, 1H, hArg OH), 6.32 (bd *J*=15.4, 1H, vTyr α-H), 6.47 (dd, *J*=15.4 and 4.7, 1H, vTyr β-H), 6.87 (part of AB-system, *J*=8.3, 2H, vTyr aryl), 7.07-7.35 (m, *J*=8.5, 13H, Phe aryl, vTyr aryl, hArg β-NH), 7.63 (d, *J*=6.0, 1H, Phe α-NH), 7.92 (d, *J*=8.4, 1H, Phe α-NH), 8.12 (d, *J*=5.7, 1H, hArg ε-NH), 8.25 (d, *J*=9.4, 1H, vTyr γ-NH) and 11.50 (bs, 1H, hArg ω-NH).

(2*S*, 5*R*, 10*S*, 13*R*, 17*S*)-*N*-[3-[5,13-Dibenzyl-10-[4-(*tert*-butyloxy)benzyl]-4, 7, 12, 15, 16, 19-hexaoxo-1, 2, 3, 4, 5, 6, 7, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 19a-octadecahydro-1*H*-3a, 6, 11, 14, 18-pentaaza-cyclopentacyclooctadecen-17-yl]propylamino[(*tert*-butyloxycarbonylimino)methyl]]-*tert*-butyloxycarbonylamine (143)

A solution of **142** (70.6 mg, 69.0 μmol), Dess-Martin periodinane (**114**) (43.9 mg, 0.103 mmol) and *t*-BuOH (8.6 μL, 91.1 μmol) in CH_2Cl_2 (1.1 mL) was stirred at room temperature for 4.5 h. Subsequently, the reaction mixture was diluted with EtOAc (2 mL) and vigorously shaken with a solution of $NaS_2O_3 \cdot 5H_2O$ (0.73 g) in saturated aqueous $NaHCO_3$ (2.0 mL), until a clear bi-phasic system was obtained. The layers were separated and the organic layer was washed with saturated aqueous $NaHCO_3$, H_2O and brine, dried (Na_2SO_4), filtrated, and concentrated *in vacuo* to give **116** as a white foam (55.3 mg, 78.5%).

^{1}H-NMR (DMSO-d_6): 1.25-1.88 (m, 7H, Pro β and γ-H, hArg γ- and δ-H), 1.28 (s, 9H, *t*-Bu), 1.40 (s, 9H, Boc), 1.49 (s, 9H, Boc), 2.00 (m, 1H, Pro β-H), 2.50-3.46 (m, 10H, Pro δ-H, hArg ε-H, Phe β-H (2x), vTyr δ-H),4.03-4.92 (m, 5H, Pro α-H, hArg α-H, Phe α-H (2x), vTyr γ-H), 5.99 (bd *J*=15.2, 1H, vTyr α-H), 6.41 (dd, *J*=15.4 and 3.6, 1H, vTyr β-H), 6.83- 7.38 (m, 14H, aryl), 8.00-8.12 (m, 2H, NH), 8.32 (m, 1H, NH), 8.49-8.62 (m, 2H, NH) and 11.50 (m, 1H, hArg ω-NH).

(2*S*, 5*R*, 10*S*, 13*R*, 17*S*)-*N*-[3-[5,13-Dibenzyl-10-[4-hydroxybenzyl]-4, 7, 12, 15, 16, 19-hexaoxo-1, 2, 3, 4, 5, 6, 7, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 19a-octadecahydro-1*H*-3a, 6, 11, 14, 18-pentaaza-cyclopentacyclooctadecen-17-yl]propylamino(imino)methyl]amine (118)

Prepared from **143** (42.1 mg, 41.2 μmol) according to the same procedure as described for the synthesis of **1b** [Chapter Five]. The crude product was purified by reverse phase HPLC; column: Waters delta-Pak C_{18} 40x100 mm (Waters 37688); mobile phase: (MeCN/H_2O, 3:2)/H_2O/phosphate buffer (0.5 M, pH= 2.1), gradient from 20:60:20 to 80:0:20 in 57 min; flow: 40 mL/min. The product was desalted on the same column (MeCN/H_2O, 3:2)/H_2O/HCl_{aq} (0.1 N), 0:80:20 15 min, then 80:18:2), and lyophilized to give **118**·1.5HCl·20H_2O (based on peptide content) as a fluffy white powder (24.0 mg, 49.5% from **143**); analytical HPLC, ≥98.5%. FAB-MS *m/z* 765 (MH^+) and 783 ($MH^+ + H_2O$). The ^{1}H-NMR spectrum was quite complicated, and indicated the presence of at least one additional compound. From the presence of a second system for δ-Pro (multipet at 3.25 ppm) we concluded that, besides the desired compound, also the ring-opened compound, H-Pro–kArg–D-Phe–vTyr–D-Phe-OH was present. HPLC analysis of the NMR sample showed the presence of four peaks (61.6, 17.3, 8.7 and 6.9% respectively). The major peak corresponds to the single peak observed for **118**.

2(*S*)-[(*tert*-Butyloxycarbonyl)amino]-6-[(benzyloxycarbonyl)amino]hexanoic acid (144)

Prepared from L-lysine according to literature procedures,[7] to give **144** as a thick oil in 91.6%.

^{1}H-NMR ($CDCl_3$): 1.37-1.97 (m, 6H, β-, γ- and δ-H), 1.45 (s, 9H, Boc), 3.18 (m, 2H, ε-H), 4.27 (m, 1H, α-H), 5.10 (bs, 2H, O$\underline{CH_2}$Ph), 5.12 (m, 1H, NH), 5.25 (m, 1H, NH) and 7.25-7.40 (m, 5H, aryl).

_N_-Methoxy, _N_-methyl-2(_S_)-[(_tert_-butyloxycarbonyl)amino]-6-[(benzyloxycarbonyl)amino]-hexanamide (145)

To a stirred solution of **144** (15.3 g, 40.1 mmol) and HOBt (9.45 g, 69.9 mmol) in CH_2Cl_2 (100 mL) at 0 °C was added DCC (8.15 g, 39.4 mmol). After 30 min, *N*-methoxy, *N*-methylamine (3.05 g, 49.9 mmol) was added and stirring was continued for 18 h at room temperature. The reaction mixture was diluted with EtOAc and filtrated to remove dicyclohexylurea. The organic solution was sequentially washed with aqueous $NaHCO_3$ (5%, 3x), H_2O (2x), aqueous $KHSO_4$ (6%, 3x), H_2O (2x) and brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo* to give, after purification by column chromatography (EtOAc/CH_2Cl_2, 3:1), **145** as a thick oil (12.7 g; 74.6%).

^{1}H-NMR ($CDCl_3$): 1.30-1.85 (m, 6H, β-, γ- and δ-H), 1.42 (s, 9H, Boc), 3.12-3.19 (m, 5H, ε-H, *N*-CH_3), 3.73 (s, 3H, *N*-OCH_3), 4.62 (m, 1H, α-H), 4.82 (m, 1H, ε-NH), 5.06 (bs, 2H, $OC\underline{H}_2Ph$), 5.18 (bd, *J*=9.0, 1H, α-NH) and 7.25-7.40 (m, 5H, aryl).

2(_S_)-[(_tert_-Butyloxycarbonyl)amino]-6-[(benzyloxycarbonyl)amino]hexanal (146)

A suspension of $LiAlH_4$ (0.96 g, 25.3 mmol) in Et_2O (100 mL) was stirred for 1 h at rt and cooled to –45 °C. Subsequently, a solution of **145** (8.47 g, 20.0 mmol) in Et_2O (20 mL) was slowly added to maintain the temperature below –35 °C. After the addition was complete stirring was continued for 5 min after which the temperature was allowed to raise to 5 °C. The reaction mixture was again cooled to –35 °C and a solution of $KHSO_4$ (5.23 g) in H_2O (14 mL) was carefully added. Finally, the reaction mixture was stirred for 1 h at room temperature, and filtrated over celite. The filtrate was sequentially washed with HCl_{aq} (1 N, 2x), saturated aqueous $NaHCO_3$ (2x), and brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo* at ambient temperature to give **146** as an oil (6.97 g; 95.4%)

^{1}H-NMR ($CDCl_3$): 1.30-1.97 (m, 6H, β-, γ- and δ-H), 1.43 (s, 9H, Boc), 3.19 (m, 2H, ε-H), 4.17 (m, 1H, α-H), 4.82 (m, 1H, ε-NH), 5.12 (bs, 2H, $OC\underline{H}_2Ph$), 5.16 (m, 1H, α-NH), 7.26-7.38 (m, 5H, aryl) and 9.54 (s, 1H, C(O)H).

1,1,1-Tris(methylthio)-3(_S_)-[(_tert_-butyloxycarbonyl)amino]-7-[(benzyloxycarbonyl)amino]-heptan-2(_R,S_)-ol (147)

n-Butyllithium (1.6 M in hexanes, 50.0 mL, 80.0 mmol) was added, over a period of 10 min to a stirred solution of tris(methylthio)methane (11.0 mL, 82.5 mmol) in THF (225 mL) at –65 °C. After 20 min a precipitate had formed. A precooled (–65 °C) solution of aldehyde **146** (6.97 g, 19.1 mmol) in THF (130 mL) was added in 30 min, upon which the precipitate dissolved. Stirring was continued for 4 h, and the reaction mixture was poured onto a stirred mixture of saturated aqueous NH_4Cl/CH_2Cl_2 (800 mL, 1:1), the layers were separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic phases were washed with brine, dried ($MgSO_4$), filtrated and evaporated to dryness *in vacuo*. Purification of the crude product by column chromatography (PE/EtOAc, 7:4) furnished **147** as an oil (diastereomers (6:1) 6.97 g; 70.2%).

^{1}H-NMR ($CDCl_3$): 1.30-1.85 (m, 6H, γ-, δ- and ε-H), 1.40 and 1.42 (2x s, 9H, Boc, diast), 2.18 and 2.19 (2x s, 9H, $C(SCH_3)_3$, diast), 3.09-3.25 (m, 3H, ω-H, OH), 3.68 and 3.89 (2x m, 1H, α-H diast), 4.02 (m, 1H, β-H), 4.90 (m, 1H, ω-NH), 5.09 (bs, 2H, $OC\underline{H}_2Ph$), 5.23 (bd, *J*=8.7, 1H, β-NH) and 7.27-7.38 (m, 5H, aryl).

Methyl 2(_R,S_)-hydroxy-3(_S_)-[(_tert_-butyloxycarbonyl)amino]-7-[(benzyloxycarbonyl)amino]-heptanoate (148)

A solution of orthothioester **147** (5.50 g, 10.6 mmol) in MeOH (190 mL) and H_2O (16.0 mL) was

vigorously stirred with $HgCl_2$ (9.82 g, 36.2 mmol) and HgO (3.24 g, 15.0 mmol) for 6 h at reflux. The reaction mixture was allowed to cool to room temperature, filtrated over celite, and the residue was thoroughly washed with CH_2Cl_2, MeOH and H_2O. The bi-phasic filtrate was separated, and the aqueous layer was extracted with CH_2Cl_2. The combined organic phases was sequentially washed with, saturated aqueous NH_4OAc (3x) and saturated aqueous NH_4Cl (2x), dried (Na_2SO_4), filtrated, and concentrated *in vacuo*, to give, after purification by column chromatography (EtOAc/PE, 2:3), **148** as a white foam (diastereomers (5:1), 2.88 g; 64.0%)

^{1}H-NMR ($CDCl_3$): 1.30-1.70 (m, 6H, γ-, δ- and ε-H), 1.38 and 1.41 (2x s, 9H, Boc, diast), 3.09-3.25 (m, 3H, ω-H, OH), 3.72 and 3.73 (2x s, 3H, OCH_3 diast), 3.98 (m, 1H, β-H), 4.11 and 4.28 (2x m, 1H, α-H diast), 4.64-4.28 (m, 2H, β-NH, ω-NH), 5.09 (bs, 2H, $OC\underline{H}_2Ph$) and 7.27-7.38 (m, 5H, aryl).

^{13}C-NMR ($CDCl_3$): 22.6 and 22.9 (δ diast), 28.1 and 28.2 ($OC(\underline{C}H_3)_3$, diast), 28.7 and 29.4 (γ, diast), 31.5 (ε), 40.5 (ω), 52.4 (β), 52.6 and 52.6 (OCH_3, diast), 66.5 ($O\underline{C}H_2Ph$), 71.9 and 73 2 (α, diast), 79.4 and 79.6 ($O\underline{C}(CH_3)_3$, diast), 127.9 (aryl), 128.3 (aryl), 136.5 (aryl-1), 155.4 and 155.9 ($\underline{C}(O)O$, Boc, diast), 156.4 ($\underline{C}(O)O$, Z), 173.2 and 173.9 ($\underline{C}(O)OCH_3$, diast).

2(*R,S*)-Hydroxy-3(*S*)-[(*tert*-butyloxycarbonyl)amino]-7-[(benzyloxycarbonyl)amino]heptanoic acid (149)

To a stirred solution of **148** (2.31 g, 5.44 mmol) in THF/MeOH/H_2O (162 mL, 4:1:1) was added $LiOH{\cdot}H_2O$ (0.69 g, 16.4 mmol). After 75 min, the pH of the reaction mixture was adjusted to pH≈ 3, and the reaction mixture was diluted with EtOAc. The layers were separated, and the organic layer was washed with aqueous $KHSO_4$ (6%, 3x), H_2O (2x), and brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo* to give **149**, as an off-white foam (diastereomers (6:1), 2.00 g; 89.5%).

^{1}H-NMR ($CDCl_3$): 1.30-1.67 (m, 6H, γ-, δ- and ε-H), 1.41 and 1.43 (2x s, 9H, Boc, diast), 3.18 (m, 2H, ω-H), 4.00 (m, 1H, β-H), 4.17 and 4.32 (2x m, 1H, α-H, diast), 4.92-5.20 (m, 4H, β-NH, ω-NH, $OC\underline{H}_2Ph$) and 7.27-7.38 (m, 5H, aryl).

Benzyl 2(*R*)-[[2(*R,S*)-hydroxy-3(*S*)-[(*tert*-butyloxycarbonyl)amino]-7-[(benzyloxycarbonyl)-amino]heptanoyl]amino]-3-phenylpropanoate (150)

Prepared from **149** (2.00, 4.87 mmol) and D-phenylalanine benzyl ester hydrochloride (1.44 g, 4.94 mmol) according to the same procedure as described for the preparation of **94** [Chapter Four]. The crude product was purified by centrifugal chromatography (CH_2Cl_2/EtOAc, 4:1), to give **150** as a white foam (diastereomers >10:1, 2.29 g, 70.7%)

^{1}H-NMR ($CDCl_3$): 1.18-1.82 (m, 6H, hLys γ-, δ- and ε-H), 1.42 (bs, 9H, Boc), 2.99-3.33 (m, 5H, hLys ω-H and OH, Phe β-H), 3.80 (m, 1H, hLys β-H), 4.06 and 4.30 (2x m, 1H, α-H, diast), 4.85-5.00 (m, 2H, hLys ω-NH, Phe α-H), 5.00-5.26 (m, 5H, hLys β-NH, $OC\underline{H}_2Ph$ 2x), 6.96-7.10 (m, 2H, Phe aryl) and 7.15-7.38 (m, 9H, aryl, Phe α-NH).

Benzyl 2(*R*)-[[2(*R,S*)-hydroxy-3(*S*)-amino-7-[(benzyloxycarbonyl)amino]heptanoyl]amino]-3-phenylpropanoate (151)

To a stirred solution of **150** (2.29 g, 3.45 mmol) in CH_2Cl_2 (60 mL) at 0 °C was added TFA (40 mL). After stirring for 2.5 h at rt, the volatiles were removed *in vacuo*. The residue was dissolved in $CHCl_3$ and washed with saturated aqueous $NaHCO_3$ (3x), and brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo* to give **151**, as a yellow oil (1.49 g; 76.5%). The ^{1}H-NMR spectrum in $CDCl_3$ was rather complex and did not allow full assignment of the signals. However, the signals for the Boc group present in the starting material had clearly disappeared.

Benzyl 2(*R*)-[[2(*R,S*)-hydroxy-3(*S*)-[[[1-(*tert*-butyloxycarbonyl)pyrrolidin-2(*S*)-yl]carbonyl]-amino]-7-[(benzyloxycarbonyl)amino]heptanoyl]amino]-3-phenylpropanoate (152)

To a stirred solution of Boc-Pro-OH[8] (0.61 g, 2.83 mmol) and HOBt (0.77 g, 5.70 mmol) in THF/DMF (18.5 mL, 3:2) at 0 °C was added 1-ethyl-3-[3'-(dimethylamino)propyl]carbodiimide hydrochloride (EDC, 0.73 g, 3.81 mmol). After 50 min, a solution of dipeptide **151** (1.49 g, 2.63 mmol) in THF (14 mL) was added. Subsequently, the reaction mixture was stirred for 18 h at room temperature. The volatiles were removed under reduced pressure, and the residue was partitioned between EtOAc and aqueous $KHSO_4$. The layers were separated, and the organic layer was washed with aqueous $KHSO_4$ (6%, 3x), H_2O (2x), aqueous $NaHCO_3$ (5%, 3x), H_2O (2x), and brine, dried (Na_2SO_4), filtrated and concentrated *in vacuo* to give, after centrifugal chromatography (CH_2Cl_2/EtOAc, 7:4), **152** as a white foam (diastereomers >10:1, 1.00 g; 51.8%).

^{1}H-NMR ($CDCl_3$): 1.12-1.66 (m, 6H, hLys γ-, δ- and ε-H), 1.41 and 1.42 (bs, 9H, Boc diast), 1.76 (m, 2H, Pro γ-H), 1.94 (m, 2H, Pro β-H), 2.98-3.22 (m, 5H, hLys ω-H and OH, Phe β-H), 3.22-3.51 (m, 2H, Pro δ-H), 3.87-4.25 (m, 3H, hLys α- and β-H, Pro α-H), 4.85 (m, 1H, Phe α-H), 5.00-5.20 (m, 5H, hLys β-NH, OC$\underline{H}_2$Ph 2x), 7.00-7.13 (m, 2H, Phe aryl), 7.14-7.39 (m, 14H, aryl, NH) and 7.46 (bd, *J*=8.3, 1H, NH). ^{13}C-NMR ($CDCl_3$): 22.9 (δ-hLys), 24.2 (b, γ-Pro), 28.1 (OC($\underline{C}H_3$)$_3$), 29.0 (γ-hLys), 39.5 (ε-hLys), 31.1 (b, β-Pro), 38.0 (β-Phe), 40.5 (ω-hLys), 46.9 (b, δ-Pro), 52.4 (α-Phe), 52.8 (β-hLys), 60.4 (b, α-Pro), 66.3 (O$\underline{C}H_2$Ph), 67.0 (O$\underline{C}H_2$Ph), 72.8 (α-hLys), 80.2 (O$\underline{C}$(CH$_3$)$_3$), 126.9 (Phe aryl-4), 127.8 (aryl), 127.9 (aryl), 128.2 (aryl), 128.3 (aryl), 128.4 (aryl), 135.0 (aryl-1 Phe), 135.6 (aryl-1), 136.6 (aryl-1), 154.9 (b, C(O)O, Boc), 156.5 (C(O)O, Z), 171.4 (C(O)O, ester), 172.4 (C(O)NH, hLys-Phe) and 173.6 (b, C(O)NH, Pro-hLys).

2(*R*)-[[2(*R,S*)-Hydroxy-3(*S*)-[[[1-(*tert*-butyloxycarbonyl)pyrrolidin-2(*S*)-yl]carbonyl]amino]-7-[(benzyloxycarbonyl)amino]heptanoyl]amino]-3-phenylpropanoic acid (153)

Prepared from **152** (0.85 g, 1.14 mmol) according to the same procedure as described for the synthesis of **97** [Chapter Four]. The crude product was purified by column chromatography (gradient from $CHCl_3$ to $CHCl_3$/MeOH, 2:1), to give **153** as a yellowish foam (0.67 g, 89.7%).

^{1}H-NMR (DMSO-d_6): 0.92-1.54 (m, 6H, hLys γ-, δ- and ε-H), 1.30 and 1.38 (2x bs, 9H, Boc rotam), 1.54-2.11 (m, 4H, Pro β- and γ-H), 2.81-3.02 (m, 4H, hLys ω-H, Phe β-H), 3.02-3.40 (m, 2H, Pro δ-H), 3.73-3.98 (m, 2H, hLys α- and β-H), 4.09 (m, 1H, Pro α-H), 4.30 (m, 1H, Phe α-H), 5.00 (m, 2H, OC$\underline{H}_2$Ph), 6.20 (m, <1H, OH), 7.05-7.41 (m, 11H, aryl, NH), 7.50 (bt, *J*=5.8, 1H, hLys ω-NH) and 7.61 (bd, *J*= 7.4, 1H, NH).

Allyl 2(*S*)-acetylamino-3-[[4(*S*)-[[2(*R*)-[[2-(*R,S*)-hydroxy-3(*S*)-[[[1-(*tert*-butyloxycarbonyl)-pyrrolidin-2(*S*)-yl]carbonyl]amino]-7-[(benzyloxycarbonyl)amino]heptanoyl]amino]-3-phenyl-propanoyl]amino]pent-2(*E*)-enoyl]amino]propanoate (154)

Prepared from tripeptide **153** (671 mg, 1.03 mmol), dipeptide **130a** (396 mg, 1.24 mmol), DiPEA (427 μL, 2.44 mmol) and TBTU (385 mg, 1.20 mmol) according to the same procedure as described for the synthesis of **110** [Chapter Four]. After aqueous work-up, the product was purified by preparative TLC (CH_2Cl_2/THF/EtOH, 24:16:1), to give **154** as a white foam (780 mg, 83.8%).

^{1}H-NMR (DMSO-d_6, 400.1 MHz): 1.00 (d, *J*=6.7, 3H, vAla δ-H), 1.06-1.48 (m, 6H, hLys γ-, δ- and ε-H), 1.31 and 1.38 (2x s, 9H, Boc rotam), 1.62-2.07 (m, 4H, Pro β- and γ-H), 1.84 (s, 3H, Ac), 2.85-3.01 (m, 4H, hLys ω-H, Phe β-H), 3.22 (m, 1H, Pro δ-H), 3.30 (m, 1H, Pro δ-H), 3.38 (m, 1H, Dpr β-H), 3.52 (m, 1H, Dpr β-H), 3.81-4.00 (m, 2H, hLys α- and β-H), 4.10 (m, 1H, Pro α-H), 4.30-4.48 (m,

2H, vAla γ-H, Dpr α-H), 4.49-4.59 (m, 3H, Phe α-H, $OC\underline{H}_2CH{=}CH_2$), 5.00 (bs, 2H, $OC\underline{H}_2Ph$), 5.25 (m, 2H, $OCH_2CH{=}C\underline{H}_2$), 5.81-5.97 (m, 3H, hLys OH, vAla α-H, $OCH_2C\underline{H}{=}CH_2$), 6.52 (dd, *J*=15.4 and 5.2, 1H, vAla β-H), 7.12-7.40 (m, 12H, Phe aryl and α-NH, Z aryl, hLys ω-H), 7.62 and 7.69 (2x d, *J*=8.4, 1H, hLys β-NH rotam), 8.11-8.20 (m, 2H, vAla γ-NH, Dpr β-NH) and 8.28 (d, *J*=8.4, 1H, Dpr α-NH).

2(*S*)-Acetylamino-3-[[4(*S*)-[[2(*R*)-[[2-(*R,S*)-hydroxy-3(*S*)-[[[1-(*tert*-butyloxycarbonyl)pyrrolidin-2(*S*)-yl]carbonyl]amino]-7-[(benzyloxycarbonyl)amino]heptanoyl]amino]-3-phenylpropanoyl]amino]pent-2(*E*)-enoyl]amino]propanoic acid (155)

To a stirred solution of **154** (728 mg, 0.791 mmol) and morpholine (1.33 mL, 15.2 mmol) in THF (50 mL) was added $Pd(PPh_3)_4$ (40.0 mg, 34.6 μmol). After 20 min the reaction was complete (checked with TLC, $THF/CH_2Cl_2/EtOH$, 25:10:1). The volatiles were removed under reduced pressure, the residue was dissolved in EtOAc and washed with aqueous $KHSO_4$ (6%, 3x), H_2O (2x), and brine. The organic solution was dried (Na_2SO_4), filtrated and concentrated *in vacuo*, to give **155**, after purification by preparative TLC (EtOAc/MeOH/HOAc, 4:1:0.5), as a sticky white foam, contaminated with HOAc (846 mg, >100%).

^{1}H-NMR (DMSO-d_6, 400 MHz: 1.00 (d, *J*=6.7, 3H, vAla δ-H), 1.05-1.45 (m, 6H, hLys γ-, δ- and ε-H), 1.30 and 1.37 (s, 9H, Boc rotam), 1.71 (bs, >3H, $C\underline{H}_3COOH$, with Pro β- and γ-H), 1.85 (m, 3H, Ac), 1.95 (s, 1H, Pro β-H), 2.82-3.00 (m, 4H, hLys ω-H, Phe β-H), 3.17-3.37 (m, 3H, Pro δ-H, Dpr β-H), 3.42 (m, 1H, Dpr β-H), 3.71-4.00 (m, 3H, hLys α- and β-H, Dpr α-H), 4.10 (m, 1H, Pro α-H), 4.39 (m, 1H, vAla γ-H), 4.53 (m, 1H, Phe α-H), 5.00 (bs, 2H, $OC\underline{H}_2Ph$), 5.94 (d, *J*=15.2, 1H, vAla α-H), 6.45 (dd, *J*= 15.2 and 4.8, 1H, vAla β-H), 7.15-7.39 (m, 11H, Phe aryl, Z aryl, hLys ω-H), 7.50 and 7.55 (2x d, *J*=8.5, 1H, NH rotam), 7.61 (d, *J*=6.4, 1H, NH), 7.81 and 7.90 (2x d, *J*=8.0, 1H, NH rotam), 7.95 (m, 1H, NH) and 8.20 (d, *J*=8.2, 1H).

2(*S*)-Acetylamino-3-[[4(*S*)-[[2(*R*)-[[2-(*R,S*)-hydroxy-3(*S*)-[[2(*S*)-(pyrrolidine)carbonyl]amino]-7-[(benzyloxycarbonyl)amino]heptanoyl]amino]-3-phenylpropanoyl]amino]pent-2(*E*)-enoyl]-amino]propanoic acid (156)

To a stirred suspension of **155** (840 mg, max 0.785 mmol + HOAc, *vide supra*) in CH_2Cl_2 (12.0 mL) at 0 °C was added TFA (8.0 mL). The resulting solution was stirred for 4 h at room temperature. Subsequently, the volatiles were removed under reduced pressure, to give **156** as a slightly coloured oil (935 mg, 100% for **156**·2.8 TFA).

^{1}H-NMR (DMSO-d_6): 0.99 (d, *J*=6.7, 3H, vAla δ-H), 1.05-1.55 (m, 6H, hLys γ-, δ- and ε-H), 1.65-1.85 (m, 3H, Pro β- and γ-H), 1.84 (s, 3H, Ac), 2.17 (m, 1H, Pro β-H), 2.81-3.04 (m, 4H, hLys ω-H, Phe β-H), 3.09-3.58 (m, 4H, Pro δ-H, Dpr β-H), 3.96-4.05 (m, 2H, hLys α- and β-H), 4.05-4.54 (m, 4H, Pro α-H, Phe α-H, vAla γ-H, Dpr α-H), 5.00 (bs, 2H, $OC\underline{H}_2Ph$), 5.90 (d, *J*=15.2, 1H, vAla α-H), 6.15 (bd, *J*=4.7, 1H, hLys OH), 6.52 (dd, *J*=15.2 and 4.8, 1H, vAla β-H), 7.12-7.40 (m, 10H, Phe aryl, Z aryl), 7.68 (d, *J*= 8.0, 1H, NH) and 8.06-8.33 (m, 5H, NH).

***(2S,5S,11S,14R,17(R,S),18S)-N*-[11-Methyl-14-benzyl-17-hydroxy-18-[[(benzyloxycarbonyl)amino]butyl]-4, 8, 13, 16, 20-pentaoxo-2,3, 4, 5, 6, 7, 8, 11, 12,13, 14, 15, 16, 17, 18, 19, 20, 20a-octadecahydro-1*H*-3a, 7, 12, 15, 19-pentaaza-cyclopentacyclononadecen-5-yl]acetamide (157)**

Prepared from **156** (430 mg, 0.364 mmol + TFA) according to the same procedure (*vide infra*) as described for the synthesis of **112** [Chapter Five]. Prior to cyclization, DMAP was added to neutralize TFA; THF was used as solvent instead of CH_2Cl_2. The product was purified by preparative TLC

(CH_2Cl_2/THF/EtOH, 120:80:1.5), to give **157** as a white foam (97.3 mg, 33.3%).
^{1}H-NMR (DMSO-d_6, 400.1 MHz): 1.00 (d, *J*=7.3, 3H, vAla δ-H), 1.20-1.55 (m, 6H, hLys γ-, δ- and ε-H), 1.78 (m, 2H, Pro γ-H), 1.81 (s, 3H, Ac), 1.88 (m, 1H, Pro β-H), 2.07 (m, 1H, Pro β-H), 2.65 (m, 1H, Dpr β-H), 2.78 (m, 1H, Phe β-H), 2.82 (m, 1H, Phe β-H), 2.98 (m, 2H, hLys ω-H), 3.31 (m, 1H, Pro δ-H), 3.56 (m, 1H, Pro δ-H), 3.96 (m, 1H, Dpr β-H), 4.03 (m, 1H, hLys α-H), 4.05-4.15 (m, 2H, hLys β-H and OH), 4.33 (m, 1H, vAla γ-H), 4.39-4.50 (m, 2H, Pro α-H, Dpr α-H), 4.60 (m, 1H, Phe α-H), 5.00 (bs, 2H, OC$\underline{H}_2$Ph), 5.88 (dd, *J*=15.4 and 2.1, 1H, vAla α-H), 6.50 (dd, *J*=15.4 and 2.2, 1H, vAla β-H), 7.10-7.39 (m, 11H, Phe aryl, Z aryl, hLys ω-NH), 7.84 (d, *J*=9.2, 1H, hLys β-NH), 8.05 (d, *J*=7.6, 1H, vAla γ-NH), 8.18 (d, *J*=7.3, 1H, Dpr α-NH), 8.26 (d, *J*=8.5, 1H, Phe α-NH) and 8.52 (bd, *J*=10.1, 1H, Dpr β-NH).

***(2S,5S,11S,14R,18S)*-*N*-[11-Methyl-14-benzyl-18-[[(benzyloxycarbonyl)amino]butyl]-4,8,13,16,17,20-hexaoxo-2,3,4,5,6,7,8,11,12,13,14,15,16,17,18,19,20,20a-octadecahydro-1*H*-3a,7,12,15,19-pentaaza-cyclopentacyclononadecen-5-yl]acetamide (158)**

Prepared from **157** (92.3 mg, 0.121 mmol) according to the same procedure as described for the synthesis of **116** [Chapter Five], to give **158** as a white foam (90.5 mg, *ca* 93%, based upon the ^{1}H-NMR-spectrum, which showed the presence of *ca* 5% starting material).
^{1}H-NMR (DMSO-d_6): 1.04 (d, *J*=7.4, 3H, vAla δ-H), 1.11-2.15 (m, 10H, Pro β- and γ-H, hLys γ-, δ- and ε-H), 1.81 (bs overlapping, 3H, Ac), 2.55-3.10 (m, 5H, hLys ω-H, Phe β-H, Dpr β-H), 3.19 (m, 1H, Pro δ-H), 3.51 (m, 1H, Pro δ-H), 3.93 (m, 1H, Dpr β-H), 4.32-4.61 (m, 5H, Pro α-H, hLys β-H, Phe α-H, vAla γ-H, Dpr α-H), 5.00 (bs, 2H, OC$\underline{H}_2$Ph), 5.79 (bd, *J*=15., 1H, vAla α-H), 6.60 (dd, *J*=15.3 and 1.6, 1H, vAla β-H), 7.07-7.43 (m, 11H, Phe aryl, Z aryl, hLys ω-NH), 8.01 (bd, *J*=8.3, 1H, NH), 8.10 (bd, *J*=9.0, 1H, NH), 8.22 (bd, *J*=6.8, 1H, NH), 8.41 (bd, *J*=8.7, 1H, NH) and 8.76 (bd, *J*=6.0, 1H, NH).

***(2S,5S,11S,14R,18S)*-*N*-[11-Methyl-14-benzyl-18-aminobutyl-4,8,13,16,17,20-hexaoxo-2,3,4,5,6,7,8,11,12,13,14,15,16,17,18,19,20,20a-octadecahydro-1*H*-3a,7,12,15,19-pentaaza-cyclopentacyclononadecen-5-yl]acetamide (119)**

Prepared from **158** (80.2 mg, 0.119 mmol) according to the same procedure (with a reaction time of 195 min instead of 85 min) as described for the synthesis of **1b** [Chapter Five]. The crude product was purified by reverse phase HPLC; column: Waters delta-Pak C_{18} 40x100 mm (Waters 37688); mobile phase: (MeCN/H_2O, 3:2)/H_2O/phosphate buffer (0.5 M, pH= 2.1), gradient from 10:70:20 to 80:0:20 in 57 min; flow: 40 mL/min. The product was desalted on the same column (MeCN/H_2O, 3:2)/ H_2O/HCl_{aq} (0.1 N), 0:80:20 15 min, then 80:18:2), and lyophilized to give **119**·1.5HCl·19H_2O (based on peptide content) as a fluffy white powder (48.8 mg, 44.5 %); analytical HPLC, ≥98.4%.
^{1}H-NMR (D_2O, 400.1 MHz): 0.94 (d, *J*=7.3, 3H, vAla δ-H), 1.38 (m, 1H, kLys δ-H), 1.48 (m, 1H, kLys δ-H), 1.57 (m, 1H, kLys γ-H), 1.70 (m, 2H, kLys ε-H), 1.78-1.92 (m, 2H, Pro β- and γ-H), 1.92-2.03 (m, 2H, Pro γ-H, kLys β-H), 1.98 (s, 3H, Ac), 2.29 (m, 1H, Pro β-H), 2.90 (t, *J*=12.0, 1H, Dpr β-H), 2.93 (m, 1H, Phe β-H), 3.02 (m, 2H, kLys ω-H), 3.07 (dd, *J*=13.1 and 5.8, 1H, Phe β-H), 3.43 (m, 1H, Pro δ-H), 3.73 (m, 1H, Pro δ-H), 4.06 (dd, *J*=11.3 and 2.5, 1H, kLys β-H), 4.19 (dd, *J*=12.9 and 5.9, 1H, Dpr β-H), 4.30 (m, 1H, vAla γ-H), 4.51 (m, 1H, vAla γ-H), 4.39-4.50 (m, 1H, Pro α-H), 4.71 (dd, *J*=9.2 and 5.9, 1H, Phe α-H), 4.61 (m, *J*=11.3 and 6.0, 1H, Dpr α-H), 5.82 (dd, *J*=15.6 and 2.4, 1H, vAla α-H), 6.65 (dd, *J*=15.6 and 2.6, 1H, vAla β-H), 7.23-7.27 (m, 2H, Phe aryl) and 7.30-7.41 (m, 3H, Phe aryl). ^{13}C-NMR (D_2O, 100 MHz): 19.4 (δ-vAla), 23.9 (C(O)$\underline{C}H_3$), 24.8 (δ-kLys), 27.3

(γ-Pro), 28.3 (γ-kLys), 29.1 (ε-kLys), 32.9 (β-Pro), 41.6 (β-Dpr), 42.0 (ω-kLys), 42.3 (β-Phe), 48.6 (γ-vAla), 51.4 (δ-Pro), 53.1 (α-Dpr), 57.3 (β-kLys), 57.5 (α-Phe), 63.3 (α-Pro), 97.1 (α-kLys), 123.9 (α-vAla), 129.9 (aryl-4 Phe), 131.4 (aryl-3 Phe), 132.0 (aryl-2 Phe), 133.3 (aryl-2 Phe), 138.4 (aryl-1 Phe), 147.7 (β-vAla), 170.4 (C(O)NH), 173.2 (C(O)NH), 173.7 (C(O)NH), 174.4 (C(O)NH), 176.0 (C(O)NH) and 176.6 (C(O)NH). FAB-MS *m/z* 626 (MH^+) and 644 ($MH^+ + H_2O$).

6.7. References and Notes

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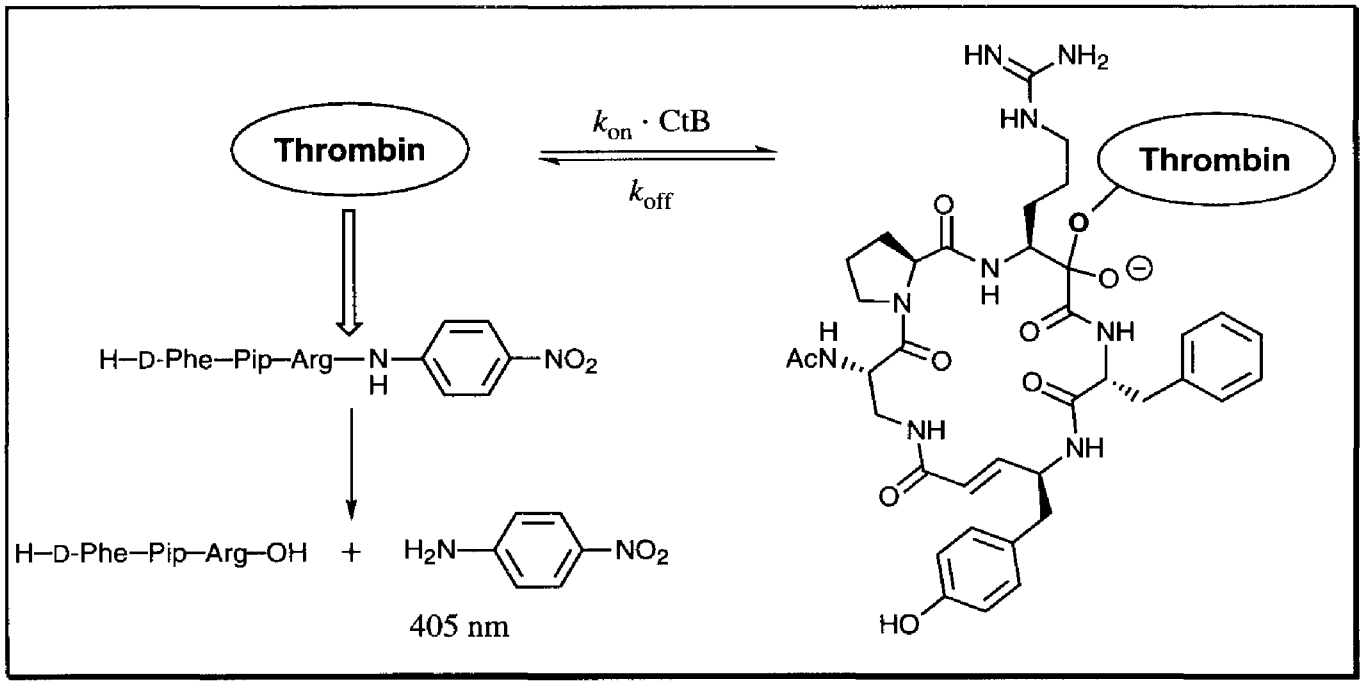
8. Prepared from L-proline and Boc-N_3[a] according to the procedure described by: Grzonka, Z.; Lammek, B. *Synthesis* **1974**, 661. a) Carpino, L.A; Giza, C.A.; Carpino, B.A. *J. A. Chem. Soc.* **1959**, *81*, 955.

CHAPTER SEVEN

Enzyme Inhibition by Cyclotheonamide B and Analogues



Abstract

Cyclotheonamide A was characterized by Lewis *et al.* as a slow-tight-binding inhibitor of several serine proteases. In this chapter an analysis of the kinetic properties of thrombin inhibition by Cyclotheonamide B is presented. The results confirm the unusual binding behaviour reported by Lewis.

Furthermore, the IC_{50}-values for thrombin inhibition by the analogues described in Chapter Six are discussed. Only part of our ideas about the molecular recognition is validated by the structure-activity relationship.

For two analogues the inhibitory activity against several other serine proteases was determined.

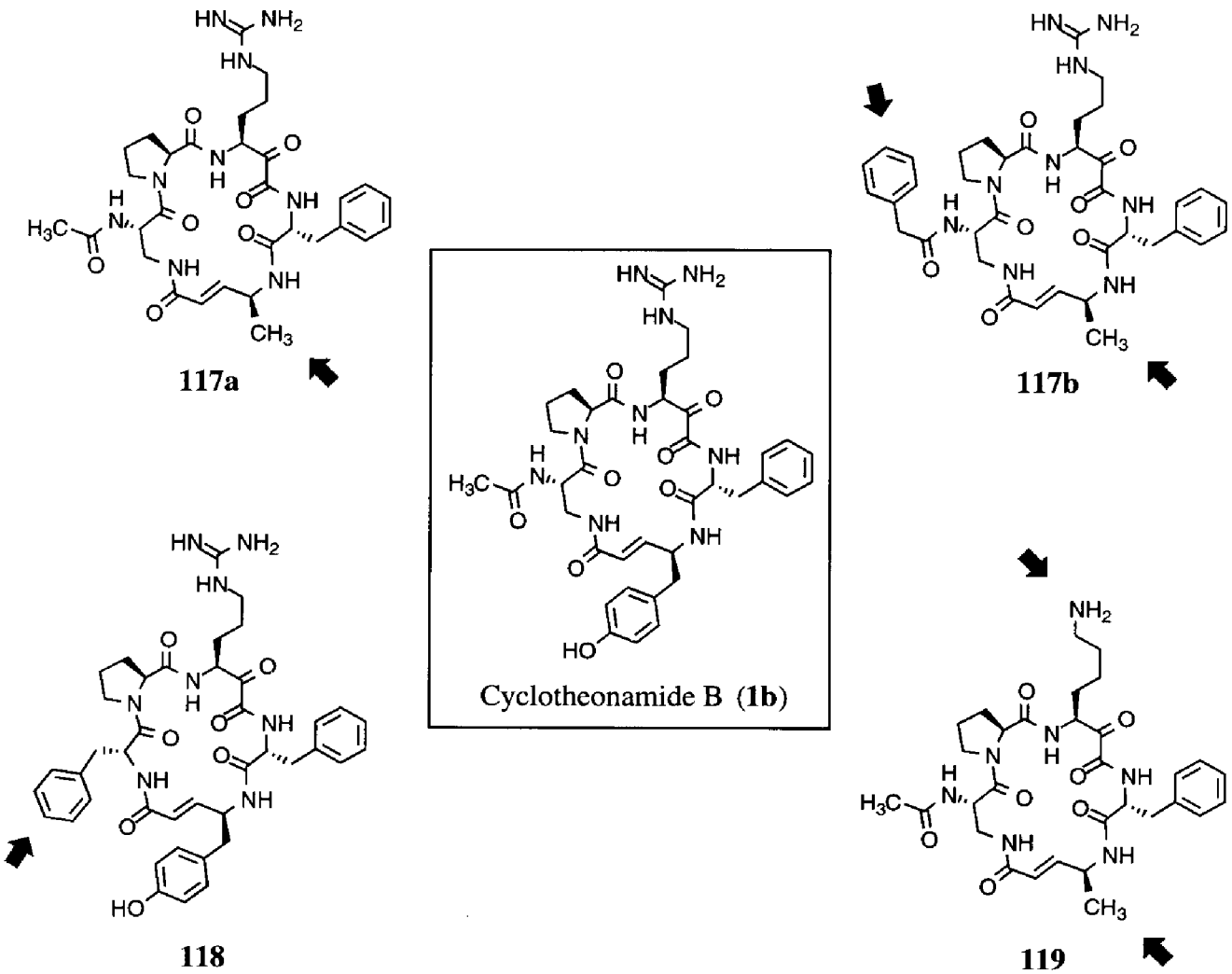
7.1. Introduction

A major reason for our interest in Cyclotheonamide stems from its ability to inhibit thrombin, a pivotal enzyme in the regulation of hemostasis and thrombosis [Chapter 1.1 and 1.2]. In their initial paper, Fusetani and Matsunaga reported an IC_{50}-value of 0.076 mg/mL for the inhibition of thrombin by natural Cyclotheonamide A, and IC_{50}-values of 0.2 mg/mL for trypsin and 0.3 mg/mL for plasmin.[1]

More elaborate studies on the natural product's biological activity by Lewis *et al.* showed that inhibition of thrombin and other trypsin-like serine proteases was a slow, time-dependent process.[2] This time-dependent inhibition explains the marked differences in K_i-values reported.[2-5] Slow binding inhibition kinetics were clearly observed when the substrate was added to an equilibrated mixture of Cyclotheonamide A and thrombin. Under conditions where thrombin was added to a mixture of substrate and inhibitor the occurrence of slow-binding inhibition was dependent on the substrate as well as on the thrombin concentration. Furthermore, Lewis *et al.* showed that Cyclotheonamide A inhibits trypsin more potently than thrombin, with K_i = 1.0 and 0.2 nM, respectively.[2]

In this chapter the enzyme inhibition data for synthetic Cyclotheonamide B (**1b**) and the four analogues described in the previous chapter (**117a,b**, **118** and **119**, Figure 7.1) are reported and discussed.

Figure 7.1. Cyclotheonamide B (**1b**) and analogues **117a,b**, **118** and **119**.

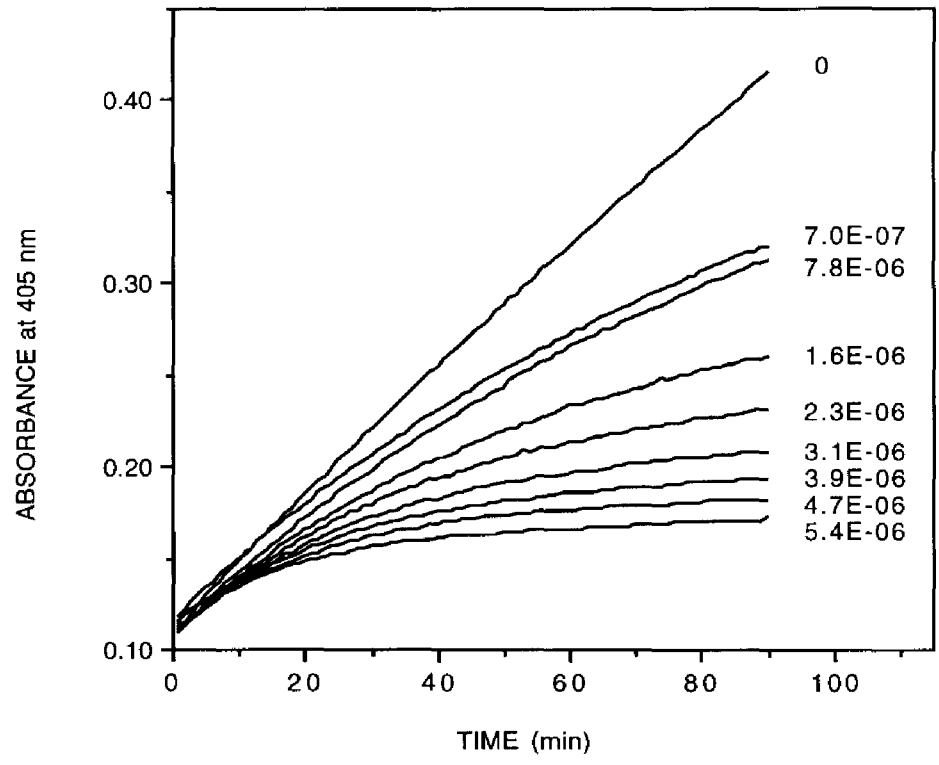


Our investigation of slow-binding enzyme kinetics for thrombin inhibition by Cyclotheonamide B is given in Section 7.2. In Section 7.3, the IC_{50}-values for inhibition of thrombin by the analogues are discussed. Finally, in Section 7.4, the selectivity of analogues **117a** and **117b** towards inhibition of other serine proteases is discussed.

7.2. Kinetic Properties of Thrombin Inhibition by Cyclotheonamide B

The anti-thrombin activity of Cyclotheonamide B was tested by monitoring (at 405 nm) the release of *p*-nitroaniline from chromogenic substrate S2238 (D-Phe–Pip–Arg-*p*-nitroanilide·2HCl) by thrombin, in the presence of different concentrations of inhibitor. Reactions were started by addition of thrombin to a mixture of Cyclotheonamide B and the substrate. The curves in figure 7.1 show the typical time-dependent inhibition of thrombin by Cyclotheonamide B and are rather similar to those reported by Lewis *et al.*.[2] They display a short-lived, pre-steady-state phase before the true steady-state is reached.

Figure 7.1. Representative progress curves showing time dependent inhibition of thrombin by Cyclotheonamide B at different concentrations.



Reactions were started by addition of human α-thrombin to a mixture of chromogenic substrate (S2232) and Cyclotheonamide B at pH= 7.4, 37 °C. Concentrations: thrombin, 0.005 IU/mL; S2238, 800 μM; Cyclotheonamide B, from 0.70 μM to 5.4 μM (see graph). Substrate depletion was insignificant over the course of the assay (*ca* 6%).

The analysis of slow-binding inhibition is based on the assumption that the kinetically significant steps in the pre-steady-state phase are those due to the binding and dissociation of the inhibitor. At high substrate concentrations (depletion is negligible during assay, <10%) and low enzyme concentrations

(only a small fraction of the inhibitor binds to the enzyme) the release of *p*-nitroaniline P from the chromogenic substrate is described by the equation:

$$[P] = V_s \cdot t - (V_s - V_0)(1 - e^{-k_{obs}})/k_{obs} \qquad (i)$$

where V_s is the final, steady-state velocity, V_0 is the initial velocity and k_{obs} is the apparent first order rate constant.[6-8] From the observed linear dependence of the observed first-order rate constant k_{obs} on the inhibitor concentration [CtB] depicted in Figure 7.2, plot **A**, it can be concluded that inhibition of thrombin by Cyclotheonamide B is a one-step process, involving a single thrombin-inhibitor complex [Scheme 7.2]. The relationship between the rate constant k_{obs} from equation *i*, and the *on* and *off* rate constants, k_{on} and k_{off} is defined by:

$$k_{obs} = k_{off} + k_{on} \cdot [CtB]/(1 + [S2238]/K_m) \qquad (ii)$$

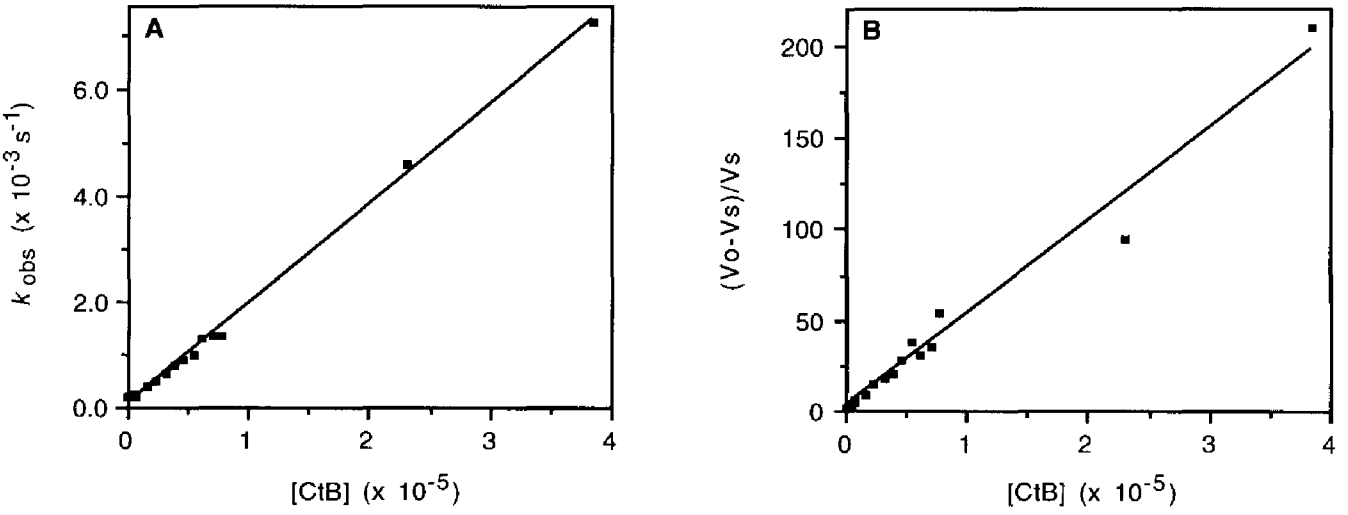
where K_m is the Michaelis-Menten constant and [S2238] is the concentration of the chromogenic substrate. In principle, both k_{on} and k_{off} can be determined from Figure 7.2. However, the intercept at the ordinate was too small to allow accurate determination of k_{off}. By plotting $(V_0 - V_s)/V_s$ against [CtB] [Figure 7.3 **B**], K_i was determined using the equation:[9]

$$(V_0 - V_s)/V_s = [CtB]/(K_i + [S2238]/K_m) \qquad (iii)$$

Hence, k_{off} was calculated from K_i and k_{on} using the relationship:

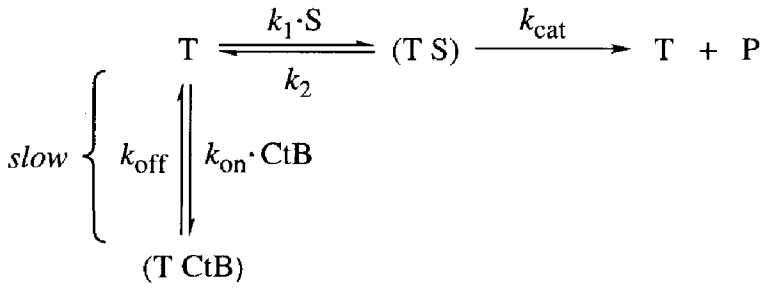
$$K_i = k_{off}/k_{on} \qquad (iv)$$

Figure 7.2. Plots of Cyclotheonamide B concentration [CtB] versus k_{obs}, and $(V_0\text{-}V_s)/V_s$.



Assay conditions are similar as those given in Figure 7.1, K_m (S2238) = 6.0 x 10^{-6}, Cyclotheonamide B concentrations range from 78 nM to 39 μM (19 data points). **A**: k_{obs} vs [CtB]: k_{on} = 2.3 x 10^4 M^{-1} s^{-1}; **B**: $(V_o - V_o)/V_o$ vs [CtB]: k_{off} = 3.3 x 10^{-5} s^{-1}, K_i= 1.5 nM.

Scheme 7.2. One-step slow-binding mechanism for thrombin (T) inhibition by Cyclotheonamide B.



k_{on} = 2.3 x 10^4 M^{-1} s^{-1}

k_{off} = 3.3 x 10^{-5} s^{-1}

K_i = 1.5 x 10^{-9} M

The K_i-value determined by us (1.5 nM) is in fair agreement with those reported by Lewis *et al.*[2] (1.0 nM) and Maryanoff *et al.*[4] (3.7 nM). Also the rate constants, k_{on} and k_{off}, are in the same range as reported by Lewis *et al.* (k_{on} = 4.7 x 10^4 $M^{-1}s^{-1}$ and k_{off} = 4.6 x 10^{-5} s^{-1}).[2] The observed k_{off}-value of 3.3 x 10^{-5} s^{-1} indicates a tight interaction of Cyclotheonamide B with thrombin; the half-life of the complex is 5.8 h. Obviously, this tight interaction can be ascribed to the covalent interaction of the keto-arginine unit with the hydroxyl group of Ser-195 of the enzyme, as was observed in the solid-state structure.[3-5]

The slow binding of Cyclotheonamide B to thrombin is reflected by the relatively low k_{on}-value of 2.3 x 10^4 $M^{-1}s^{-1}$. For a diffusion-controlled association this value is approximately 10^8 $M^{-1}s^{-1}$.[10] The molecular rational for the observed slow binding is still obscure. Since Cyclotheonamide's electrophilic keto group is fully hydrated in aqueous solution, we originally anticipated that slow binding might be caused by the time elapse necessary for the dissociation of this *gem*-diol to a (protonated) keto group which would allow formation of the covalent enzyme-inhibitor complex. However, the observation of Maryanoff *et al.* that a Cyclotheonamide analogue with a hydroxyl instead of a keto group also exhibits slow-binding properties is inconsistent with this dissociation hypothesis.[4] Therefore, it seems more likely that a series of conformational adjustments of Cyclotheonamide upon interaction with the enzyme is responsible for the observed slow-binding properties.[4]

7.3. Inhibition of Thrombin by Analogues of Cyclotheonamide B

The anti-thrombin activity of the Cyclotheonamide analogues was also tested by monitoring the cleavage of chromogenic substrate S2238 by thrombin, in presence of different inhibitor concentrations. Inhibition experiments, using a standard protocol, indicated that inhibition of thrombin by analogues **117a,b**, **118** and **119** is also a time-dependent process.

In Table 7.1, the inhibitory activity of these analogues and of Cyclotheonamide B are presented as IC_{50}-values. These IC_{50}-values, *i.e.* the concentrations at which the thrombin activity was reduced to 50%, were calculated from the absorbances measured at 90 min reaction time. The enzyme and inhibitor concentrations used in the standard protocol did not allow determination of the kinetic constants (see Section 7.2 and Experimental).

The results given in Table 7.1 learned us the following. Our idea with regard to the role of the vinylogous tyrosine residue present in Cyclotheonamide seems to be valid [Chapter 2.3]. Analogue

117a with a methyl group instead of a hydroxybenzyl group was found to inhibit thrombin with nearly the same IC_{50}-value (entry 2) as was found for the parent compound (entry 1). Thus, it can be concluded that the interaction of the hydroxyphenyl group with the insertion loop of thrombin adds only very little to the total binding.
More surprising were the activities of the analogues having a hydrophobic group at the P_3 position. Analogue **117b**, with a phenylacetyl group at the α-amino group of Dpr (entry 3), is only slightly more potent than Cyclotheonamide B. We had expected that this hydrophobic phenylacetyl group would interact strongly with the S_3 pocket, thereby increasing the potency a 100 to 1000-fold, comparable to the observations of Bajusz *et al.* for a series of tripeptide aldehydes.[11]
Also the second analogue with an additional hydrophobic group (*i.e.* **118**) does not exhibit a higher activity (entry 4) than Cyclotheonamide B. To the contrary, a 3-fold lower potency is observed. However, this might be due to the instability of this compound [Chapter 6.3].
Replacement of the arginine side chain by an aminobutyl moiety (*i.e.* **119**) results in a 50-fold lower inhibitory activity (entry 5) compared to **117a**.

Table 7.1. IC_{50}-values for thrombin inhibition by Cyclotheonamide B and analogues.

	Compound	IC_{50} (μM)
1	Cyclotheonamide B (**1b**)	0.64
2	**117a**	0.84
3	**117b**	0.39
4	**118**	1.6
5	**119**	42

In summary, of the analogues prepared, **117b** is the most active one. The gain in activity with respect to Cyclotheonamide B, although very small, is probably due to an improved, albeit incomplete, interaction with the S_3 pocket of thrombin. Our results are in agreement with the study of Maryanoff *et al.* who reported the anti-thrombin activities of a series of Cyclotheonamide analogues with similarly modified vinylogous tyrosine and diaminopropanoic acid residues.[5]

7.4. Selectivity of 117a,b Towards Trypsin-Like Serine Proteases

The activity of analogues **117a** and **117b** against three other serine proteases was also determined. This was done by monitoring the cleavage of a chromogenic substrate by the enzyme. For the various enzymes different chromogenic substrates were used (see Experimental). The IC_{50}-values were calculated from the absorbances measured at 90 min [Table 7.2].
Inspection of the IC_{50}-values presented in Table 7.2 shows that Cyclotheonamide B (**1b**) is most active against trypsin, as was also demonstrated for Cyclotheonamide A by Lewis *et al.*.[2] However, the activity of **1b** against plasmin was also slightly higher than against thrombin; this is in contrast to the

findings reported for Cyclotheonamide A.[2] Furthermore, it is seen that the inhibitory activity of analogues **117a** and **117b** against the various serine proteases hardly differs from the activity observed for Cyclotheonamide B (**1b**).

Table 7.2. Inhibitory activity (IC_{50}, μM) of **1b**, **117a** and **117b** against four related serine proteases.

	Enzyme	**1b**	**117a**	**117b**
1	Thrombin	0.64	0.84	0.39
2	Trypsin	0.041	0.035	0.027
3	Plasmin	0.38	-	0.18
4	Factor Xa	0.85	0.76	0.37

7.5. Conclusions

The slow-tight-binding enzyme kinetics, reported by Lewis *et al.*[2] for authentic Cyclotheonamide A, were also observed clearly for synthetic Cyclotheonamide B. The association rate for the thrombin-Cyclotheonamide B complex is four orders of magnitude lower than expected for a diffusion-controlled reaction. The reason for this slow binding is still unclear. The notion that slow binding is connected to the time elapse necessary for the dissociation of the *gem*-diol (hydrated keto-group) to a keto group which is susceptible for reacting with the enzyme, is inconsistent with the observations of Maryanoff *et al.*, who reported time-dependent inhibition of thrombin for a Cyclotheonamide A analogue with a hydroxyl group instead of a keto group.[5] Probably, a series of conformational adjustments of Cyclotheonamide upon interaction with the enzyme is responsible for the observed slow-binding properties.

The structure activity relationship for inhibition of thrombin by our series of cyclopentapeptides is still rather obscure. As anticipated, the hydroxybenzyl group hardly contributes to the binding of Cyclotheonamide B to thrombin, since replacement of the hydroxybenzyl group by a methyl group does not significantly lower the inhibitory activity. However, attempts to increase the potency of the natural product by introducing a hydrophobic phenylacetyl group were, much to our surprise, unsuccessful. Since analysis of the solid-state structure of the Cyclotheonamide A-thrombin complex clearly indicates that this hydrophobic group is able to occupy the vacant S_3 pocket, an explanation for this observation is still unclear. We are still puzzled by the discrepancy between our expectations based on molecular modelling studies involving the thrombin-Cyclotheonamide B complex, and the activities displayed by the analogues prepared. It is obvious that more compounds have to be prepared in order to clarify the structure activity relationship.

Also, the activities of analogues **117a** and **117b** against related serine proteases do not differ much from the activities observed for Cyclotheonamide B itself. Both compounds display a much higher activity against trypsin than thrombin.

From the biological data reported in this chapter it becomes once more clear that the Pro–kArg–D-Phe motif is responsible for the molecular interaction of Cyclotheonamide with thrombin and several other serine proteases. Modification of the vTyr–Dpr dipeptide does not effect the activity, whereas introduction of a lysine instead of an arginine residue causes a drop in potency.

Further efforts towards the development of more selective, cyclic keto-arginine based thrombin inhibitors should therefore focus on the role of the D-Phe residue, a residue that has not yet been addressed. More suggestions for a second generation of Cyclotheonamide analogues are discussed in the concluding chapter [Chapter Eight].

7.6. Experimental

The determination of the inhibitory activity of Cyclotheonamide B and analogues against several serine-proteases and of the accompanying enzyme kinetics were performed at the Department of Vascular Pharmacology of N.V. Organon.

Anti-thrombin assay, determination of IC_{50}-values

To a solution of S2238 ((D-Phe–Pip–Arg-p-nitroanilide·2HCl, 240 μM) and inhibitor (0 and 1.0 μM to 3.0 mM) in 0.1 M NaCl, 0.05 M Tris, pH 7.4 at 37 °C, was added a solution of thrombin (0.2 IU/mL, Centraal Laboratorium Bloedtransfusiedienst, Amsterdam) in 0.1 M NaCl, 0.05 M Tris, pH 7.4, 0.3% PEG (M_r 6000). The mixture was stirred thoroughly and the increase in absorbance (405 nm) was measured every minute for a period of 90 min (microtiter plate reader, Flow Laboratories).
The IC_{50}-values, *i.e.* the concentrations at which the thrombin activity was reduced to 50%, were calculated from the absorbances measured at 90 min .

Anti-thrombin assay, determination of kinetic constants

The antithrombin activity was assayed as described above (IC_{50}-assay), using a higher substrate concentration (800 μM) and a lower enzyme concentration (0.005 IU/mL). Progress curves were analyzed using the slow-binding kinetics.[6-9]

Inhibition of other serine protease, determination of IC_{50}-values

The inhibition of trypsin, plasmin and Factor Xa was assayed as described for thrombin, using the following chromogenic substrates: trypsin (156 ng/mL, Sigma T 1005), S2222 (benzoyl-Ile–Glu–Gly–Arg-*p*-nitroanilide·HCl); plasmin (2.2 nkat/mL, Kabi), S2251 (H-D-Val–Leu–Lys-*p*-nitroanilide·2HCl, Kabi) and bovine Factor Xa (2.13 nkat/mL, Kabi), S2222 (benzoyl-Ile–Glu–Gly–Arg–*p*-nitro–anilide·HCl).

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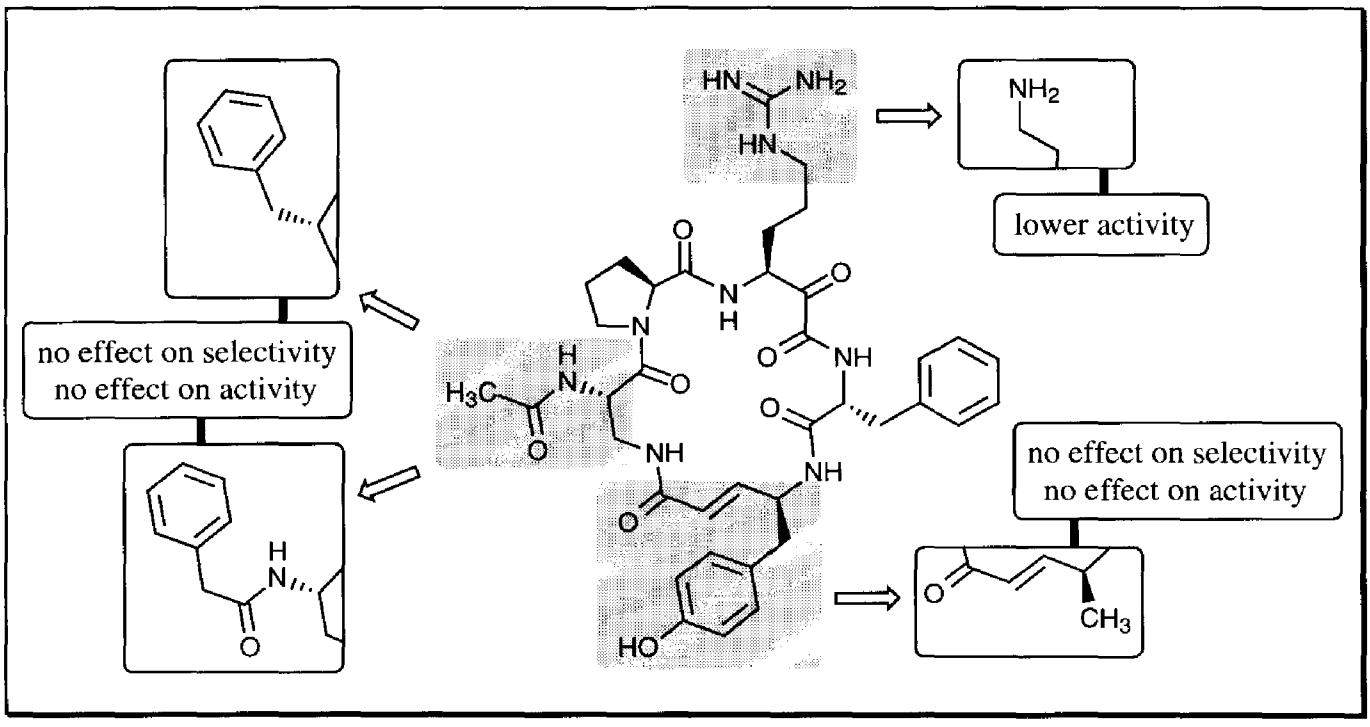
CHAPTER EIGHT

Summary and Conclusions



Abstract

The convergent strategy set out in Chapters Two to Five using conventional benzyl-, *t*-butyl- and allyl-based protecting groups in combination with a straightforward synthesis of the α-hydroxy-β-homoarginine building block provided an efficient route to both Cyclotheonamide B and a series of analogues.

In this chapter, this approach is summarized and compared to other published syntheses.

In addition, the activity of the analogues prepared is briefly discussed. The biological data for these analogues only partially confirm our ideas about the molecular interaction of Cyclotheonamide with thrombin.

Some suggestions for the design of a second generation of analogues, aiming at the improvement of potency and selectivity are given at the end of this chapter.

8.1. Synthetic Chemistry

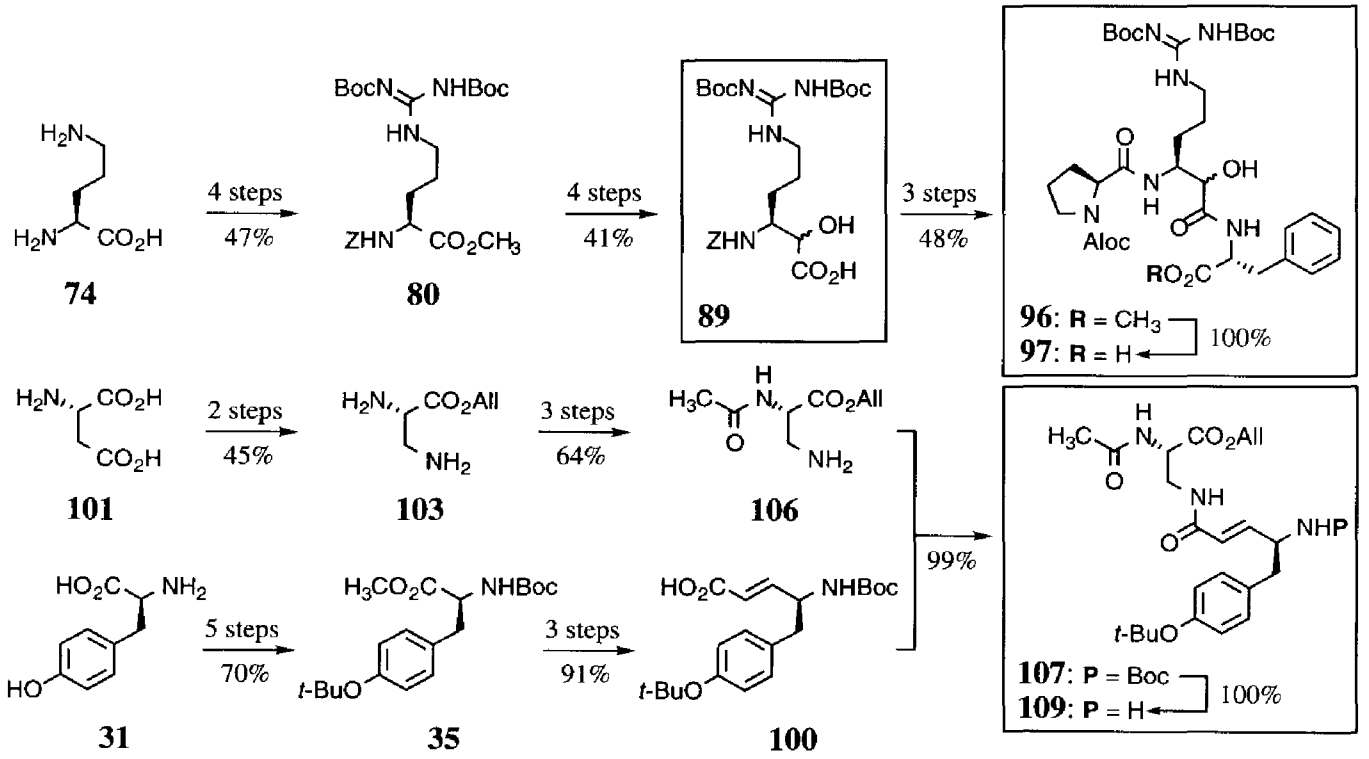
At the time we completed our total synthesis of Cyclotheonamide B, four syntheses had already been reported.[1-4] A summary of these syntheses was presented in Chapter Two. Here follows a summary of our [3 + 2] fragment-condensation approach which was laid out in Chapters Three to Five.

The synthesis of the key intermediates, *i.e.* Segment A (**97**) and Segment B (**109**), and their elaboration into Cyclotheonamide B is summarized in Scheme 8.1. Starting compound for the preparation of the α-hydroxy-β-homoarginine moiety, the predominant part of Segment A (**97**), was N^{α}-Z, $N^{\omega,\omega'}$-(bis-Boc)arginine methyl ester (**80**), prepared in four steps from L-ornithine (**74**) [Chapter Three]. Reduction of **80** to the corresponding aldehyde and subsequent addition of tris(methylthio)methyllithium, followed by treatment with $HgO/HgCl_2$ in aqueous methanol, yielded an orthogonally protected α-hydroxy methyl ester. Upon hydrolysis of this ester under standard conditions (LiOH 3 equiv, THF/MeOH/H_2O, 4:1:1, rt, 72 min) a considerable amount of cyclic carbamate was formed, due to intramolecular nucleophilic attack by the hydroxyl group at the benzyloxycarbonyl group. Decreasing the reaction time to 12 min prevented this side-reaction and gave α-hydroxy acid **89** as the only product.

Coupling (DCC/HOBt) of **89** with D-phenylalanine methyl ester followed by hydrogenolysis to remove the Z group and coupling (TBTU) with *N*-Aloc proline, gave tripeptide **96** [Chapter Four]. Selective deprotection of the *C*-terminus of **96**, to give Segment A (**97**), was achieved by saponification (LiOH, rt, 12 min). A longer reaction time caused hydrolysis of the Pro-Arg peptide bond.

For the synthesis of Segment B (**109**), the constituent amino acid were prepared as follows [Chapter Four]. Allyl L-2,3-diaminopropanoate (**103**) prepared from L-aspartic acid (**101**), was reacted with Boc_2O/TEA under high-dilution conditions to give mainly the mono N^{β}-Boc derivative of **103**.

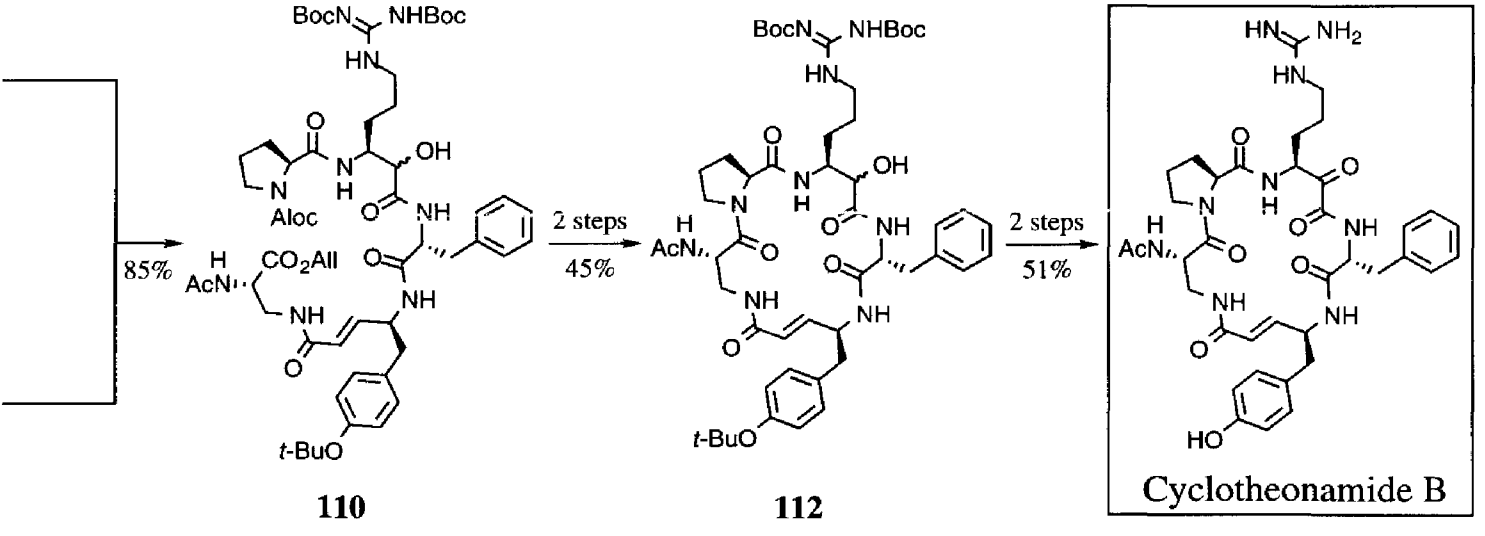
Scheme 8.1. Synthesis of Cyclotheonamide B according to Ottenheijm *et al.*.[5]



The crude reaction mixture was treated with acetyl chloride yielding, after purification by chromatography and removal of the Boc group with an etheral solution of HCl, allyl N^{α}-acetyl 2,3-diaminopropanoate (**106**).

The tyrosine-derived α,β-unsaturated γ-amino acid **100** was prepared by a Wadsworth-Emmons olefination of the aldehyde derived from *N*-Boc, *O*-*t*-butyl tyrosine methyl ester (**35**), which in turn was synthesized from L-tyrosine (**31**). The Wittig-type olefination gave in quantitative yield the ethyl ester (with exclusively the *E*-geometry), which, after hydrolysis to give **100**, was coupled (TBTU) with **106**, to furnish dipeptide **107**. Treatment of **107** with TFA gave the corresponding *O,N*-deprotected dipeptide. Unfortunately, it was found that the conditions for oxidation of an α-hydroxy-β-homoarginine moiety to the corresponding α-keto carboxylic acid derivative were incompatible with the presence of a free phenolic hydroxyl group. Consequently, we faced the challenge to achieve selective *N*-Boc removal in the presence of an aryl *t*-butyl ether. Treatment of **107** with conventional acids in different concentrations and different solvents did not bring about the desired selectivity. However, selective removal of the *N*-Boc group in **107** could be achieved by treatment with trimethylsilyl trifluoromethanesulfonate/2,6-lutidine to give Segment B (**109**) in quantitative yield.

Coupling of Segment A (**97**) with Segment B (**109**) using TBTU afforded smoothly the fully protected pentapeptide **110** [Chapter Five]. Treatment of **110** with 7 mol% of $Pd(PPh_3)_4$, in the presence of a 50-fold excess of morpholine, resulted in simultaneous removal of the *C*-terminal allyl group and the *N*-terminal Aloc group, to give, after purification by chromatography, the *N*-ethyl morpholine-salt of the *C,N*-terminal deprotected pentapeptide. Ring closure of the latter was effected under dilution conditions by treatment with TBTU/HOBt/DMAP and gave protected cyclopentapeptide **112** in 61% yield. Oxidation of the hydroxyl group with the Dess-Martin periodinane (24 h, 40 °C) in the presence of *t*-butyl alcohol, followed by *O,N*-deprotection with TFA/thioanisole, and subsequent HPLC-purification afforded Cyclotheonamide B in 51% yield.



overall: 1.8% (from **80**)
17 steps

The route presented in Scheme 8.1 was also used to prepare the four analogues [Chapter Six] depicted in Figure 8.1. Compared to the synthesis of the natural product, the only major difference observed during the preparation of these analogues were the yields of the cyclization steps. It was found that the yields of the cyclopentapeptides was dependent on the number, and the position of the aromatic groups present in the linear peptide, suggesting some kind of pre-organization through aromatic interactions prior to ring closure.

Analogue **118** (an 18-membered macrocycle) was found to be unstable in aqueous solution and to be contaminated with the seco-peptide H-Pro–kArg–D-Phe–vTyr–Dpr(Ac)-OH. This analogue was also less stable towards the conditions required for the oxidation of the intermediate hydroxy amide.

The only report to date discussing the synthesis and biological activity of Cyclotheonamide analogues appeared but very recently.[6] In their series of analogues, Maryanoff *et al.* also prepared an analogue in which the hydroxybenzyl group of vTyr was replaced by a methyl group (*i.e.* **117a**) and an analogue with a phenylacetyl group at N^{α} of Dpr (as in **117b**).

8.2. A Comparison of the Different Routes to Cyclotheonamide

Our [3+2] fragment-condensation approach towards Cyclotheonamide B is rather similar to the one reported by Maryanoff *et al.*[3] [Chapter Two]. However, compared to the synthesis of Maryanoff and also to the other reported syntheses, our protecting group strategy is more straightforward and uses only conventional benzyl-, *t*-butyl and allyl-based groups.

No exchange of protecting groups was necessary, in contrast to the syntheses of Schreiber[1] and of Wipf.[2] The hydroxyl group was left unprotected during our entire synthesis, whereas Maryanoff *et al.*,[3] Wipf *et al.*,[2] and Shiori *et al.*[4] use a temporary protecting group to block the hydroxyl group, on the one hand to prevent formation of cyclic carbamates (Maryanoff and Wipf), and on the other hand to allow selective oxidation of their carboxyl synthon (Shiori).

A major difference is also the protection of the guanidine moiety. Whereas in the other routes a N^{ω}-arylsulfonyl protecting group (Ts or Mtr) is used, sometimes in combination with a Boc group (Schreiber and Wipf), we employ another and relatively new guanidino protection, *i.e.* two Boc groups at the ω, ω'-position of arginine.

Except for the synthesis of Shiori *et al.*,[4] in all syntheses the α-hydroxy β-homoarginine building block was obtained by addition of a carboxylate anion equivalent to an arginine-derived aldehyde. The second unusual, and hitherto unknown amino acid, *viz.* vTyr, was obtained in an equal fashion (Wittig methodology) in all five syntheses.

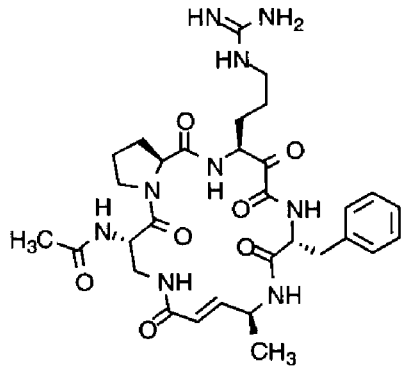
The overall yields (starting from a protected arginine derivative) for the five different syntheses are: Schreiber *et al.*,[1] 0.8% (22 steps); Wipf *et al.*,[2] 1.8% (21 steps); Maryanoff *et al.*,[3] 0.8% (17 steps), Shiori *et al.*,[4] 12% (15 steps) and Ottenheijm *et al.*,[5] 3.8% (13 steps).

All five routes are useful for the preparation of Cyclotheonamide analogues in which the amino acid residues are systematically modified. However, especially our synthesis and the synthesis of Maryanoff *et al.* are devised for the convenient synthesis of analogues with a hydrophobic substituent at the Dpr unit.[3]

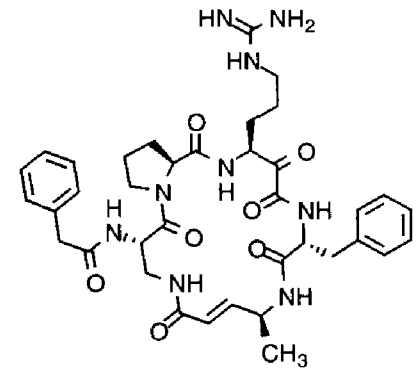
8.3. Enzyme Inhibition

Both the issues of potency towards inhibition of thrombin and of selectivity towards inhibition of related serine proteases were investigated using the four analogues of Cyclotheonamide B prepared [Chapter Seven].

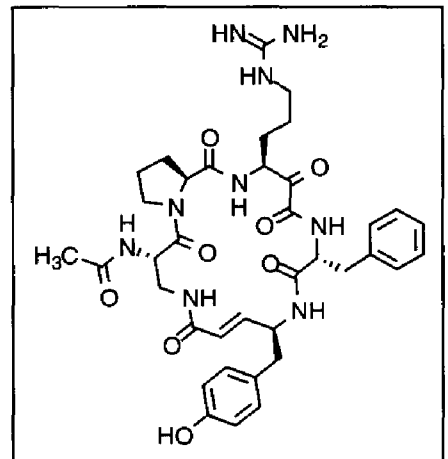
Figure 8.1. Cyclotheonamide B (**1b**) and analogues **117a,b**, **118** and **119**.



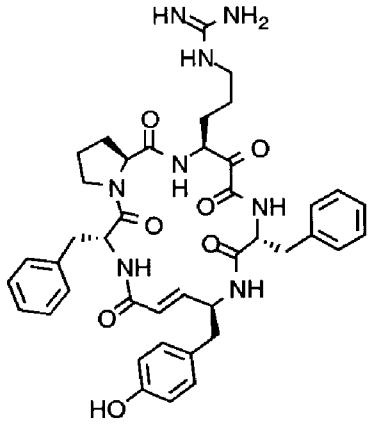
117a



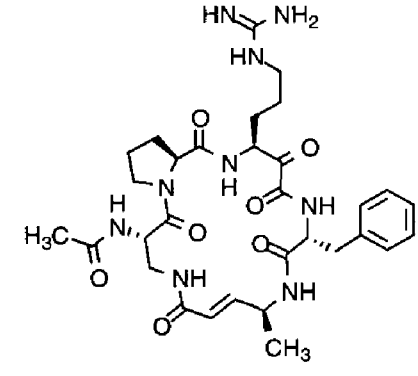
117b



Cyclotheonamide B (**1b**)



118



119

Although Maryanoff *et al.* reported that the hydroxybenzyl group of Cyclotheonamide interacted with the 60-insertion loop of thrombin,[3,6,7] we were unable to detect this interaction in the X-ray structure deposited in the protein data base. In order to clarify the role of the hydroxybenzyl group we prepared analogue **117a** in which this group was replaced by a methyl group. Since thrombin inhibition by **117a** was not significantly different from the inhibition found for Cyclotheonamide B (**1b**), it must indeed be concluded that the contribution of the hydroxybenzyl-insertion loop interaction to the total binding, if present, is negligible small.

A further modification we put forward on the basis of molecular modelling studies was the introduction of a hydrophobic group at the Dpr unit in Cyclotheonamide B. It was our firm belief that this substituent would occupy the S_3 pocket, which is left vacant in the Cyclotheonamide A-thrombin

complex. Because the phenyl group of D-Phe in the PPACK-thrombin complex was shown to interact with this S_3 pocket [Chapter One], analogue **118**, containing a D-Phe residue instead of a Dpr residue, was prepared. In a second analogue bearing a hydrophobic group, *i.e.* **117b**, the N^{α}-acetyl moiety of the Dpr residue was replaced by a phenylacetyl group. In the latter analogue the phenyl group is more extended from the core of the cyclopeptide. However, to our surprise and disappointment, both analogues inhibit thrombin almost equally well as Cyclotheonamide B. The only reasonable explanation we can give at this moment is that the phenyl group is unable to properly occupy the S_3 pocket, probably due to the fact that the spacer which attaches the phenyl group to the macrocycle is too short.
Analogue **112**, in which the arginine side chain was replaced by an aminobutyl group (lysine), displays a significantly lower activity, thus illustrating once more the superiority of the guanidinopropyl group for binding to the S_1 pocket.

Cyclotheonamide was reported to inhibit a variety of related serine proteases, *e.g.* thrombin, trypsin and plasmin. However, although not only the solid-state structure of the Cyclotheonamide A-thrombin complex but also that of the Cyclotheonamide A-trypsin complex was available, suggestions to improve the selectivity in favour of thrombin could not be put forward since the steric fit in both complexes is rather comparable. Modification of the amino acid residues of Cyclotheonamide might gives some clues about the side chains relevant for interaction with these other serine proteases. Thus, two analogues were tested on several related serine proteases, including trypsin.
The results of the enzyme inhibition experiments were quite unexpected. Both analogues **117a** and **117b** were as effective as Cyclotheonamide B in the inhibition of trypsin, plasmin and Factor Xa [Chapter Seven].

8.4. Suggestions for Further Investigation

Based on the biological data for inhibition of thrombin and other serine proteases by Cyclotheonamide B and the first generation of analogues prepared, some suggestions for further investigations aiming at improving the potency and, especially, the selectivity towards thrombin inhibition can be put forward.
From the data presented it can be concluded that solely the Pro–kArg–D-Phe moiety is responsible for molecular recognition of Cyclotheonamide by the enzymes. However, the precise role of the D-Phe residue needs to be further investigated, as interactions with the S' pockets are not yet clearly defined.[8]

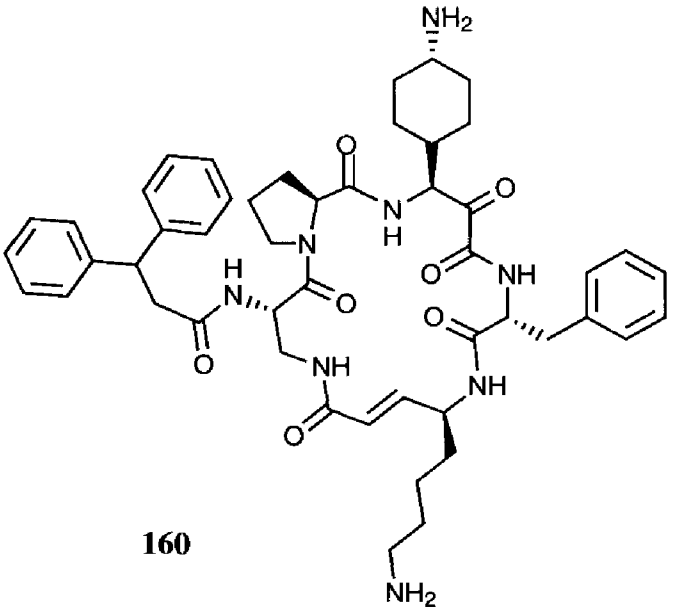
Introduction of a larger hydrophobic group or a hydrophobic group with a larger spacer might bring about the desired interaction with the S_3 pocket of thrombin. Since trypsin does not have such a pronounced S_3 pocket, introduction of a larger substituent might have a favourable effect on the selectivity. A second possibility to increase this interaction of the phenyl group in **117b** and **118** is to increase the ring size of the macrocycle, since a larger ring is more likely to allow a close contact between the phenyl group and the S_3 pocket. Along this line of reasoning, linear sequences containing a keto-arginine moiety might also be considered.

The major difference between thrombin and the related proteases, however, is the 60-insertion loop. By creating an interaction between the inhibitor and this insertion loop, the selectivity as well as the activity is likely to benefit. A basic amino acid, *i.e.* a (vinylogous) lysine residue instead of the

vTyr unit might give such an interaction with Asp-60E of the insertion loop.
Further modification of the arginine side chain might also lead to more selective inhibitors; Brady *et al.* recently showed that replacement of the lysine side chain of a keto-lysine-containing tripeptide (Me-D-Phe–Pro–kLys-NHMe) by a *trans*-4-aminocyclohexyl group resulted in very potent and selective thrombin inhibitor ($K_{i\ (thrombin)}$= 0.09 nM, $K_{i\ (trypsin)}$= 1151 nM).[9] Similarly, replacement of the lysine side chain in **119** by this aminocyclohexyl group might also lead to improved selectivity towards thrombin inhibition.

Combination of the above suggestions for effective modifications of Cyclotheonamide leads to structure **160** [Figure 8.2], which is expected to be rapidly accessible through the synthetic methodology presented in this thesis.

Figure 8.2. A second-generation Cyclotheonamide analogue.



160

8.5. References and Notes

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APPENDIX

Synthesis of β-Homoarginine Derivatives

A.1. Introduction

For the preparation of des-oxo Cyclotheonamide analogue **120** [Chapter Six, Scheme 6.1], we searched for an efficient route towards β-homoarginine, a β-amino acid derivative. Several synthetic routes to β-amino acids have been reported.[1] Of these, the most direct one is the Arndt-Eistert homologation of α-amino acids which gives the corresponding homologous β-amino acids or esters with retention of configuration.[2]

As outlined in this appendix, we developed a new method for the preparation of β-homoarginine-containing dipeptides based on Arndt-Eistert methodology [Section A.2].[3] Furthermore, we were also able to extend a β-homoarginine dipeptide into a tripeptide [Section A.3]. However, elaboration of this tripeptide into **120** was severely hampered by the choice of guanidine protecting groups.

Our investigation towards the synthesis of **120** was initiated at the start of our Cyclotheonamide project. At that time we were still using bis-Z protected arginine derivative **32** [Scheme A.2, see also Chapter Three] as a starting material for the preparation Cyclotheonamide. Therefore, we also used this derivative for the studies on the preparation of des-oxo Cyclotheonamide.

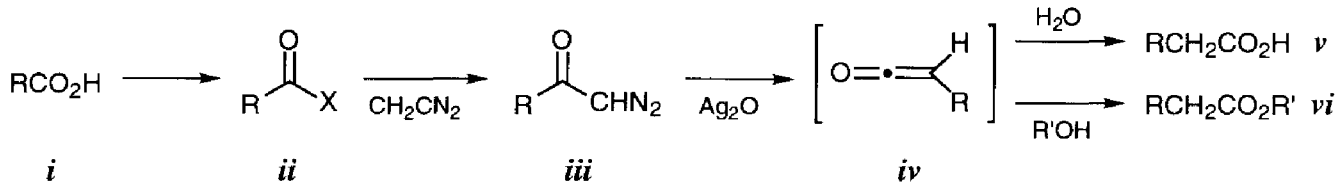
When Tulinsky firmly established that the presence of the keto-arginine unit is a prerequisite for the biological activity of Cyclotheonamide,[4] our interest in the des-oxo analogue **120** faded, and we stopped our investigations towards this compound.

Nevertheless, the newly developed method for the preparation of β-homoarginine-containing dipeptides proved to be widely applicable as will be demonstrated in Section A.2.

A.2. A New and Facile Synthesis of β-Homoarginine-Containing Dipeptides

For the preparation of the β-homoarginine building block required for the preparation of tripeptide **167** [Scheme A.3] we employed the Arndt-Eistert synthesis [Scheme A.1]. In this synthesis, a carboxylic acid (*i*) is converted to its homologue (*v*) or homologous ester (*vi*) by a Wolff-rearrangement of the intermediate diazomethyl ketone (*iii*). The latter is prepared by the reaction of an activated carboxylic acid derivative (*ii*) with diazomethane. The rearrangement of *iii* occurs upon treatment with Ag_2O or with silver benzoate/TEA, or under photochemical conditions, to give a transient ketene (*iv*) which is intercepted by H_2O to give the homologated carboxylic acid *v*, or by an alcohol to give the corresponding ester (*vi*).

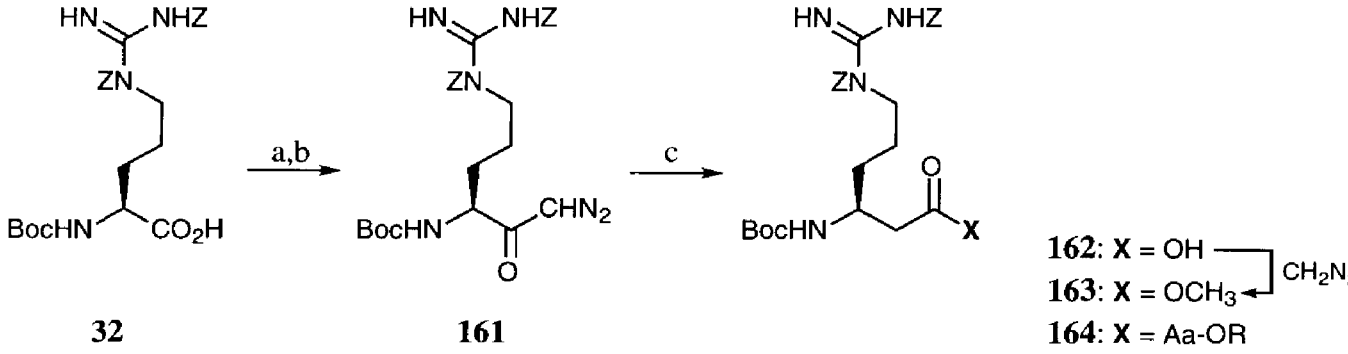
Scheme A.1. Homologation of carboxylic acids: the Arndt-Eistert synthesis.



Conversion of arginine derivative **32** into the diazomethyl ketone proved to be quite straightforward and gave **161** in 83% yield [Scheme A.2].[5] Compound **161** was used for the subsequent Wolff-rearrangement which, in view of the base-labile nature of the N^{δ}-Z group [Chapter 3.2], was carried out photochemically instead of by treatment with H_2O and Ag_2O or with silver benzoate/TEA. Thus, irradiation (300 nm) of a solution of **161** and methanol in dioxane gave β-homoarginine methyl ester **163** in 66% yield. Alternatively, **163** could also be prepared by trapping the intermediate ketene with water to give carboxylic acid **162**, and subsequently reaction with diazomethane.

Homoarginine derivative **162** can be used in standard peptide coupling reactions in order to prepare β-homoarginine containing peptides. However, we reasoned that direct peptide formation might be feasible by trapping the ketene derived from **161** with a second amino acid derivative. Indeed, photolysis of **161** in the presence of an amino ester yielded the corresponding β-homoarginine containing dipeptide ester **164**.

Scheme A.2. Synthesis of β-homoarginine derivatives.



a) *i*-butyl chloroformate, TEA, CH_2Cl_2, –20 °C, 15 min; b) CH_2N_2, 5 °C, 22 h, 85% (two steps); c) H-**X**, MeCN, 22 h, hv, 30 °C, **X** = OH, 55%, **X** = OCH_3, 66%, and **X** = Aa-OR, 45-76% (Aa = amino acid, see Table A.1).

The method proved widely applicable and so a series of dipeptides could be prepared by irradiation of a 0.025 M solution of **161** in dry MeCN in the presence of 1.2 equiv of an amino ester hydrochloride and 1.2 equiv of TEA [Table A.1].
It is noteworthy that this reaction proceeds also satisfactory with the hindered valine methyl ester (entry c) and the less nucleophilic proline methyl ester (entry e). In nearly all cases a precipitate was formed during the reaction; filtration gave the pure dipeptide. A second crop could be obtained after aqueous work-up of the filtrate and subsequent crystallization or chromatography.

Table A.1. Dipeptides **164** prepared by irradiation of **161** in the presents of amino esters (H-Aa-OR).

164	Aa	R	Yield (%)[a]	Total (%)[b]
a	D-Phenylalanine	CH_2Ph	-[c]	66
b	D-Phenylalanine	CH_3	55	64
c	L-Valine	CH_3	42	65
d	L-Tryptophan	CH_3	35	45
e	L-Proline	CH_3	-[d]	59
f	β-Alanine	CH_3	68	77

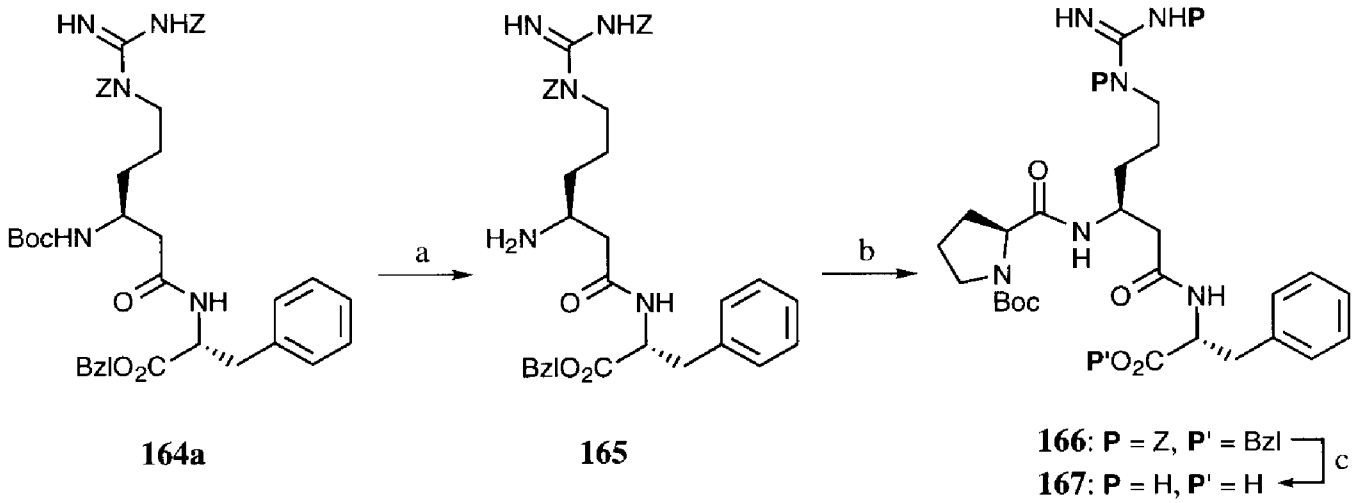
[a]Yield of dipeptide precipitated during reaction. [b]Total yield, after work-up of the filtrate and crystallization or chromatography. [c]Precipitate not isolated; the reaction mixture was worked up and purified by chromatography. [d]No precipitate was formed.

Recently, a paper on the preparation of β-amino acid-containing dipeptides was published by Seebach *et al.*[6] Following the same line of reasoning as we did, the authors used amino esters as nucleophilic reaction partners in the Arndt-Eistert homologation of α-amino acids. However, instead of irradiation, silver benzoate/TEA was employed to effect the Wolff-rearrangement. Furthermore, their method does not allow the use of amino ester hydrochlorides and failed to give a tryptophan containing dipeptide.

A.3. Synthesis of a Tripeptide Key Fragment Containing a β-Homoarginine Unit

Starting compound in the synthesis of the β-homoarginine-containing tripeptide was **164a** [Scheme A.3]. Cleavage of the *N*-Boc group to yield **165** and subsequent coupling of the latter with *N*-Boc proline gave the protected tripeptide **166**. The conditions employed for the deprotection of the carboxyl group of **166** (H_2/Pd, MeOH/H_2O/HCl, 0 °C) caused also removal of the Z groups and gave **167** in 63% yield starting from **164a**.
Attempts to further elaborate **167** into Cyclotheonamide analogue **120** were unsuccessful due to problems arising from the presence of an unprotected guanidino group (see also Chapter Three).
However, in principle, elaboration of **167** into **120** should be feasible, *e.g.* by introducing new guanidino protecting groups in **167** (*e.g.* Adoc groups, see Chapter Three). Moreover, we know now that N^{α}-Z, $N^{\omega,\omega'}$-bis(Boc)arginine (**73**), from which Cyclotheonamide and analogues were successfully prepared, would have been a more successful starting material than arginine derivative **32**.

Scheme A.3. Synthesis of tripeptide **167**.



164a **165** **166**: P = Z, P' = Bzl; **167**: P = H, P' = H

a) TFA/CH_2Cl_2 (3:2), 3 h, aqueous work-up with $NaHCO_3$, 100%; b) *N*-Boc proline/EDC/HOBt/DiPEA, THF, 21 h, 68%; c) H_2/Pd/C, MeOH/H_2O/HCl, 0 °C, 93%.

A.4. Conclusion

The methodology developed for the preparation of β-homoarginine-containing dipeptides, using amino esters as nucleophilic reaction partners in the Arndt-Eistert homologation of arginine, proved to be a facile and general method for the preparation of homologous dipeptides.
The β-homoarginine–D-phenylalanine dipeptide **164a**, prepared by this method, was successfully extended to a tripeptide key intermediate required for the preparation of des-oxo Cyclotheonamide B **120**. However, elaboration of this tripeptide into **120** was unsuccessful, due to problems arising from the selected protecting group strategy.

A.5. Experimental

Detailed general experimental information is given in Chapter 3.6.

1-Diazomethyl-2(*S*)-[(*tert*-butyloxycarbonyl)amino]-5-[[imino[(phenylmethoxycarbonyl)-amino]methyl](phenylmethoxycarbonyl)amino]pentanone (161)
To a stirred solution of **32** (4.43 g, 7.96 mmol) and TEA (1.11 mL, 7.97 mmol) in CH_2Cl_2 (40 mL) at –15 °C was added dropwise isobutyl chloroformate (1.04 mL, 8.04 mmol). After 20 min at –15 °C an ice-cold solution of diazomethane (excess, prepared as described in Chapter Three) in CH_2Cl_2 (100 mL, containing Na_2SO_4) was added dropwise. Stirring was continued for 1 h, after which the reaction mixture was stored overnight at 4 °C. Subsequently, the reaction mixture was flushed with nitrogen to remove the excess of diazomethane. The resulting solution was washed with aqueous $NaHCO_3$ (5%),

H_2O and brine, dried (Na_2SO_4), and concentrated *in vacuo*, to give, after purification by column chromatography (CH_2Cl_2/EtOAc, 23:2), **161** as a yellow solid (3.79 g, 84.0%); mp 130-131 °C.
^{1}H-NMR ($CDCl_3$): 1.40 (s, 9H, Boc), 1.60-1.72 (m, 4H, β- and γ-H), 3.95 (bd, *J*=8.0, 2H, δ-H), 4.18 (m, 1H, α-H), 5.08 and 5.16 (AB-system, *J*=12.0, 2H, OC$\underline{H}_2$Ph), 5.22 (s, 2H, OC$\underline{H}_2$Ph), 5.49 (bs, 1H, CHN_2), 5.61 (bd, 1H, *J*=8.0, α-NH), 7.25-7.42 (m, 10H, aryl), 9.29 and 9.43 (2x bs, 2H, ω-and ω'-NH). ^{13}C-NMR ($CDCl_3$): 24.7 (γ), 28.3 (OC($\underline{C}H_3$)$_3$), 28.6 (β), 44.2 (δ), 54.1 (CHN_2), 56.9 (α), 67.0 (O$\underline{C}H_2$Ph), 68.9 (O$\underline{C}H_2$Ph), 79.6 (O$\underline{C}$(CH$_3$)$_3$), 128.0 (aryl), 128.1 (aryl), 128.3 (aryl), 128.5 (aryl), 128.7 (aryl), 128.8 (aryl), 134.5 (aryl-1), 136.6 (aryl-1), 155.4 (C(O)O, Z), 155.7 (C(O)O, Boc), 160.6 (C=N), 163.6 (C(O)O, Z) and 194.1 (ketone). FAB-HRMS: calcd for $[C_{28}H_{34}N_6O_7 + H]^+$ 567.2567, found 567.2573.

3(*S*)-[(*tert*-Butyloxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)amino]methyl]-(phenylmethoxycarbonyl)amino]hexanoic acid (162)

A stirred solution of **161** (200 mg, 0.353 mmol) in 1,4-dioxane/H_2O (12.0 mL, 2:1) was irradiated (300 nm; 16 lamps, 12 Watt each) for 21 h at 30 °C. Subsequently, the turbid reaction mixture was partitioned between CH_2Cl_2 and H_2O. The aqueous layer was extracted with CH_2Cl_2 and the combined organic layers were washed with brine, dried (Na_2SO_4), filtrated, and concentrated *in vacuo*, to give, after purification by preparative TLC ($CHCl_3$/MeOH, 97:3), **162** as a viscous oil (109 mg, 55.6%). ^{1}H-NMR ($CDCl_3$): 1.35-1.75 (m, 4H, γ- and δ-H), 1.40 (s, 9H, Boc), 2.49 (bs, 2H, α-H), 3.78-4.05 (m, 3H, β- and ε-H), 5.11 (m overlapping, 1H, β-NH), 5.14 (bs, 2H, OC$\underline{H}_2$Ph), 5.23 (bs, 2H, OC$\underline{H}_2$Ph), 7.22-7.45 (m, 10H, aryl), 9.29 and 9.42 (2x bs, 2H, ω-and ω'-NH). ^{13}C-NMR ($CDCl_3$): 24.5 (δ), 28.3 (OC($\underline{C}H_3$)$_3$), 30.9 (γ), 38.9 (α), 44.3 (ε), 47.1 (β), 67.0 (O$\underline{C}H_2$Ph), 68.9 (O$\underline{C}H_2$Ph), 79.4 (O$\underline{C}$(CH$_3$)$_3$), 127.9 (aryl), 128.0 (aryl), 128.4 (aryl), 128.8 (aryl), 128.9 (aryl), 134.6 (aryl-1), 136.9 (aryl-1), 155.5 (C(O)O, Z), 155.9 (C(O)O, Boc), 160.5 (C=N), 163.7 (C(O)O, Z) and 176.0 (C(O)OH).

Methyl 3(*S*)-[(*tert*-Butyloxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)amino]-methyl](phenylmethoxycarbonyl)amino]hexanoate (163)

Prepared from **37** (180 mg, 0.318 mmol) according to the same procedure as described for the synthesis of **162**, using 1,4-dioxane/MeOH (17 mL, 2:15) instead of 1,4-dioxane/H_2O. Purification by preparative TLC (CH_2Cl_2/EtOAc, 12:1) yielded **163** as a colourless solid (119 mg, 65.5%); mp 89-91 °C.
^{1}H-NMR ($CDCl_3$): 1.36-1.78 (m, 4H, γ- and δ-H), 1.42 (s, 9H, Boc), 2.47 (bd, *J*=6.7, 2H, α-H), 3.63 (s, 3H, OCH_3), 3.79-4.03 (2x m, 3H, β- and ε-H), 5.06 (bd, 1H, *J*=10.0, β-NH), 5.13 (bs, 2H, OC$\underline{H}_2$Ph), 5.23 (bs, 2H, OC$\underline{H}_2$Ph), 7.25-7.45 (m, 10H, aryl), 9.28 and 9.43 (2x bs, 2H, ω- and ω'-NH). ^{13}C-NMR ($CDCl_3$): 24.5 (δ), 28.3 (OC($\underline{C}H_3$)$_3$), 31.0 (γ), 38.9 (α), 44.3 (ε), 47.3 (β), 51.6 (OCH_3), 66.9 (O$\underline{C}H_2$Ph), 68.8 (O$\underline{C}H_2$Ph), 79.2 (O$\underline{C}$(CH$_3$)$_3$), 127.7 (aryl), 127.8 (aryl), 128.3 (aryl), 128.4 (aryl), 128.8 (aryl), 129.0 (aryl), 134.6 (aryl-1), 136.9 (aryl-1), 155.3 (C(O)O, Z), 155.9 (C(O)O, Boc), 160.6 (C=N), 163.8 (C(O)O, Z) and 172.0 (C(O)OCH_3). FAB-HRMS: calcd for $[C_{29}H_{38}N_4O_8 + H]^+$ 571.2768, found 571.2765.

Benzyl 2(*R*)-[[3(*S*)-[(*tert*-butyloxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)-amino]methyl]amino]hexanoyl]amino]-3-phenylpropanoate (164a)

To a stirred solution of D-phenylalanine benzyl ester hydrochloride[7] (0.74 g, 2.54 mmol) in MeCN (90 mL) and TEA (353 μL, 2.54 mmol) was added **161** (1.20 g, 2.12 mmol). The solution was irradiated (300 nm), with 16 lamps of 12 Watt each, at a temperature of 30 °C. After a few minutes the evolution

of N_2 was observed. After 22 h, during which a precipitate had formed, the reaction mixture was partitioned between CH_2Cl_2 and H_2O. The aqueous layer was extracted with CH_2Cl_2 (2x) and the combined organic layers were washed with aqueous citric acid (1N), H_2O and brine, dried (Na_2SO_4), filtrated, and concentrated under reduced pressure, to give, after purification by column chromatography (CH_2Cl_2/EtOAc, 9:1), **164a** as a white powder (1.11 g, 65.9%); mp 145-148 °C.
^{1}H-NMR ($CDCl_3$): 1.38 (s, 9H, Boc), 1.12-1.62 (m, 4H, hArg γ- and δ-H), 2.29 and 2.79 (2x m, 2H, hArg α-H), 3.77 and 3.09 (2x m, 2H, Phe β-H), 3.48-3.80 (m, 3H, hArg β- and ε-H), 4.87 (m, 1H, Phe α-H), 4.98-5.15 (m, 4H, OC$\underline{H}_2$Ph 2x), 5.17 and 5.23 (AB system, 2H, *J*=13.3, OC$\underline{H}_2$Ph), 5.89 (bd, 1H, *J*=9.6, hArg β-NH), 6.63 (bd, 1H, *J*= 8.4 Hz, Phe α-NH), 6.90-7.17 (m, 5H, Phe aryl), 7.20-7.43 (m, 15H, Z aryl), 9.26 and 9.35 (2x bs, 2H, ω- and ω'-NH). ^{13}C-NMR ($CDCl_3$): 24.6 (δ-hArg), 28.4 (OC($\underline{C}H_3$)$_3$), 29.6 (γ-hArg), 37.4 (α-hArg), 37.5 (β-Phe), 44.3 (ε-hArg), 47.4 (β-hArg), 52.8 (α-Phe), 66.7 (O$\underline{C}H_2$Ph), 67.1 (O$\underline{C}H_2$Ph), 68.8 (O$\underline{C}H_2$Ph), 78.9 (O$\underline{C}$(CH$_3$)$_3$), 126.8 (aryl-4 Phe), 128.0 (aryl), 128.2 (aryl), 128.3 (aryl), 128.4 (aryl), 128.5 (aryl), 128.7 (aryl), 128.8 (aryl), 128.9 (aryl), 134.7 (aryl-1), 135.2 (aryl-1), 135.9 (aryl-1), 136.8 (aryl-1), 155.5 (C(O)O, Z), 155.7 (C(O)O, Boc), 160.4 (C=N), 163.4 (C(O)O, Z), 171.4 and 171.6 ($\underline{C}$(O)OBzl and C(O)NH). FAB-HRMS: calcd for $[C_{44}H_{51}N_5O_9 + H]^+$ 794.3765, found 794.3733.

Methyl 2(*R*)-[[3(*S*)-[(*tert*-butyloxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)-amino]methyl]amino]hexanoyl]amino]-3-phenylpropanoate (164b)
Prepared from **161** (4.63 g, 8.17 mmol) and D-phenylalanine methyl ester hydrochloride[8] (2.07 g, 9.60 mmol) according to the same procedure as described for the synthesis of **164a**. However, after 18 h, the reaction mixture was stored at 4 °C for 6 h. Subsequently, the precipitate was collected by filtration to give pure **164b** as a white powder (3.25 g; 55.4%). The filtrate was worked up as described for **164a** and the residue was crystallized to give a second crop of **164b** (0.49 g; 8.4%; total yield: 63.8%); mp 166-167 °C.
^{1}H-NMR ($CDCl_3$): 1.12-1.64 (m, 4H, hArg γ- and δ-H), 1.40 (s, 9H, Boc), 2.30 and 2.79 (2x m, 2H, hArg α-H), 3.72 and 3.05 (2x m, 2H, Phe β-H), 3.49-3.79 (m, 3H, hArg β- and ε-H), 3.63 (s, 3H, OCH_3), 4.81 (m, 1H, Phe α-H), 5.05 and 5.18 (AB-system, *J*=12.0, 2H, OC$\underline{H}_2$Ph), 5.20 (bs, 2H, OC$\underline{H}_2$Ph), 5.90 (bd, 1H, *J*=10.0, hArg β-NH), 6.60 (bd, 1H, *J*=8.3 Hz, Phe α-NH), 6.93-7.18 (m, 5H, Phe aryl), 7.20-7.45 (m, 10H, Z aryl), 9.28 and 9.38 (2x bs, 2H, ω- and ω'-NH). ^{13}C-NMR ($CDCl_3$): 24.4 (δ-hArg), 28.3 (OC($\underline{C}H_3$)$_3$), 29.5 (γ-hArg), 37.3 (α-hArg), 37.5 (β-Phe), 44.2 (ε-hArg), 47.3 (β-hArg), 52.1 (OCH_3), 52.7 (α-Phe), 66.6 (O$\underline{C}H_2$Ph), 68.7 (O$\underline{C}H_2$Ph), 78.8 (O$\underline{C}$(CH$_3$)$_3$), 126.8 (aryl-4 Phe), 127.7 (aryl), 127.9 (aryl), 128.0 (aryl), 128.2 (aryl), 128.3 (aryl), 128.3 (aryl), 128.7 (aryl), 128.7 (aryl), 134.6 (aryl-1), 135.9 (aryl-1), 136.7 (aryl-1), 155.3 (C(O)O, Z), 155.6 (C(O)O, Boc), 160.3 (C=N), 163.3 (C(O)O, Z), 171.5 and 171.9 ($\underline{C}$(O)OCH_3 and C(O)NH). FAB-HRMS: calcd for $[C_{38}H_{47}N_5O_9 + H]^+$ 718.3453, found 718.3419.

Methyl 2(*R*)-[[3(*S*)-[(*tert*-butyloxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)-amino]methyl]amino]hexanoyl]amino]-4-methylpentanoate (164c)
Prepared from **161** (1.24 g, 2.19 mmol) and valine methyl ester hydrochloride[8] (0.43 g, 2.54 mmol) according to the same procedure as described for the synthesis of **164a**. After 18 h, the reaction mixture was stored overnight at –20 °C. The precipitate was collected by filtration to give pure **164c** as a white powder (0.61 g; 41.6%). The filtrate was worked up as described for **164a**, the residue was purified by centrifugal chromatography (CH_2Cl_2/EtOAc, 85:15) to give a second crop of **164c** (0.34 g;

23.6%; total yield: 65.2%); mp 138-139 °C.
^{1}H-NMR ($CDCl_3$): 0.80 (bd, *J*=6.6, 6H, Val γ-H), 1.28-1.72 (m, 4H, hArg γ- and δ-H), 1.40 (s, 9H, Boc), 1.98 (m, 1H, Val β-H), 2.36 and 2.77 (2x m, 2H, hArg α-H), 3.63 (s, 3H, OCH_3), 3.66-4.08 (m, 3H, hArg β- and ε-H), 4.33 (m, 1H, Val α-H), 5.00-5.23 (m, 4H, $OC\underline{H}_2Ph$ 2x), 5.80 (bd, 1H, *J*=9.1, hArg β-NH), 6.48 (bd, 1H, *J*=8.4 Hz, Phe α-NH), 6.90-7.17 (m, 5H, Phe aryl), 7.20-7.43 (m, 10H, Z aryl), 9.30 and 9.39 (2x bs, 2H, ω- and ω'-NH). ^{13}C-NMR ($CDCl_3$): 17.9 (γ-Val), 18.7 (γ-Val), 24.7 (δ-hArg), 28.3 ($OC(\underline{C}H_3)_3$), 29.9 (γ-hArg), 30.6 (β-Val), 38.2 (α-hArg), 44.5 (ε-hArg), 47.8 (β-hArg), 51.9 (OCH_3), 57.2 (α-Val), 66.8 ($O\underline{C}H_2Ph$), 68.7 ($O\underline{C}H_2Ph$), 78.9 ($O\underline{C}(CH_3)_3$), 127.8 (aryl), 127.9 (aryl), 128.2 (aryl), 128.3 (aryl), 128.4 (aryl), 128.7 (aryl), 134.5 (aryl-1), 136.5 (aryl-1), 155.4 (C(O)O, Z), 155.8 (C(O)O, Boc), 160.5 (C=N), 163.6 (C(O)O, Z), 171.5 and 172.0 ($\underline{C}(O)OCH_3$ and C(O)NH). FAB-HRMS: calcd for $[C_{34}H_{47}N_5O_9 + H]^+$ 670.3452, found 670.3504.

Methyl 2(*R*)-[[3(*S*)-[(*tert*-butyloxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)-amino]methyl]amino]hexanoyl]amino]-3-indolylpropanoate (164d)
Prepared from **161** (0.90 g, 1.59 mmol) and tryptophan methyl ester hydrochloride[8] (0.53 g, 2.08 mmol) according to the same procedure as described for the synthesis of **164a**. After irradiation for 23 h the reaction mixture was stored overnight at –20 °C. The precipitate was collected by filtration to give pure **164d** as an off-white powder (0.42 g; 34.9%). The filtrate was worked up as described for **164a** and the residue was purified by centrifugal chromatography (CH_2Cl_2/EtOAc, 4:1) to give a second crop of **164d** (0.12 g;10.0%; total yield: 44.9%); mp 116-119 °C.
^{1}H-NMR ($CDCl_3$): 1.10-1.69 (m, 4H, hArg γ- and δ-H), 1.40 (s, 9H, Boc), 2.23 and 2.50 (2x m, 2H, hArg α-H), 3.10 and 3.23 (2x m, 2H, Trp β-H), 3.60-3.86 (m, 3H, hArg β- and ε-H), 3.63 (s, 3H, OCH_3), 4.85 (m, 1H, Trp α-H), 5.00-5.22 (m, 4H, $OC\underline{H}_2Ph$ 2x), 5.39 (m, 1H, hArg β-NH), 6.32 (m, 1H, Trp α-NH), 6.85 (d, *J*= 2.1, 1H, indolyl-2), 7.08 (m, 2H, indolyl-5,6), 7.19-7.50 (m, 12H, indolyl-4,7 and aryl Z), 8.31 (m, 1H, indolyl-NH), 9.28 and 9.38 (2x bs, 2H, ω- and ω'-NH). ^{13}C-NMR ($CDCl_3$): 24.8 (δ-hArg), 27.4 (β-Trp), 28.3 ($OC(\underline{C}H_3)_3$), 29.9 (γ-hArg), 39.2 (α-hArg), 44.3 (ε-hArg), 47.7 (β-hArg), 52.1 (OCH_3), 52.4 (α-Trp), 66.7 ($O\underline{C}H_2Ph$), 68.8 ($O\underline{C}H_2Ph$), 79.0 ($O\underline{C}(CH_3)_3$), 109.7 (indolyl-3), 111.3 (indolyl-7), 118.3 (indolyl-6), 119.4 (indolyl-4), 121.9 (indolyl-5), 122.7 (indolyl-2), 127.3 (indolyl-3a), 127.8 (aryl Z), 128.2 (aryl Z), 128.3 (aryl Z), 128.7 (aryl Z), 128.8 (aryl Z), 134.5 (aryl-1 Z), 136.0 (indolyl-7a), 135.6 (aryl-1 Z), 136.8 (aryl-1), 155.4 (C(O)O, Z), 155.7 (C(O)O, Boc), 160.5 (C=N), 163.5 (C(O)O, Z), 170.8 and 172.3 ($\underline{C}(O)OCH_3$ and C(O)NH). FAB-HRMS: calcd for $[C_{40}H_{48}N_6O_9 + H]^+$ 757.3561, found 757.3541.

Methyl 1-[3(*S*)-[(*tert*-butyloxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)amino]-methyl]amino]hexanoyl]pyrrolidine-2(*S*)-carboxylate (164e)
Prepared from **161** (1.34 g, 2.36 mmol) and proline methyl ester hydrochloride[8] (0.56 g, 3.37 mmol) according to the same procedure as described for the synthesis of **164a**. After irradiation for 20 h no precipitate was present. The reaction mixture was worked up as described for **164a**. The crude product was purified by centrifugal chromatography (CH_2Cl_2/EtOAc, 85:15) to give **164e** (0.93 g; 59.0%); mp 84-86 °C.
^{1}H-NMR ($CDCl_3$): 1.34-2.19 (m, 8H, hArg γ- and δ-H, Pro β- and γ-H), 1.37 (s, 9H, Boc), 2.40 and 2.63 (2x m, 2H, hArg α-H), 3.33 and 3.52 (2x m, 2H, Pro δ-H), 3.67 and 3.70 (2x s, 3H OCH_3 rotam), 3.77-4.05 (m, 3H, hArg β- and ε-H), 4.38 (m, 1H, Pro α-H), 5.09 and 5.17 (AB system, 2H, *J*=15.0, $OC\underline{H}_2Ph$), 5.17 (m, 2H, $OC\underline{H}_2Ph$), 5.73 (bd, 1H, *J*=9.0, hArg β-NH), 7.22-7.47 (m, 10H, Z aryl), 9.28

and 9.43 (2x bs, 2H, ω- and ω'-NH). ^{13}C-NMR ($CDCl_3$): 24.5 (δ-hArg), 25.4 and 25.5 (γ-Pro rotam), 28.2 ($OC(\underline{C}H_3)_3$), 28.9 (γ-hArg), 30.4 and 30.9 (β-Pro rotam), 37.3 (α-hArg), 44.2 (ε-hArg), 47.4 (β-hArg), 46.9 and 47.1 (δ-Pro rotam), 51.9 and 52.3 (OCH_3 rotam), 58.3 and 59.1 (α-Pro rotam), 66.6 ($O\underline{C}H_2Ph$), 68.6 ($O\underline{C}H_2Ph$), 78.5 ($O\underline{C}(CH_3)_3$), 127.5 (aryl), 127.7 (aryl), 128.0 (aryl), 128.1 (aryl), 128.4 (aryl), 128.5 (aryl), 134.5 (aryl-1), 136.7 (aryl-1), 155.4 (C(O)O, Z), 155.6 (C(O)O, Boc), 160.4 (C=N), 163.6 (C(O)O, Z), 169.9 and 172.3 ($\underline{C}(O)OCH_3$ and C(O)N). FAB-HRMS: calcd for $[C_{34}H_{45}N_5O_9 + H]^+$ 668.3296, found 668.3308.

Methyl 3-[[3(*S*)-[(*tert*-butyloxycarbonyl)amino]-6-[[imino[(phenylmethoxycarbonyl)amino]-methyl]amino]hexanoyl]amino]propanoate (164f)

Prepared from **161** (1.37 g, 2.42 mmol) and β-alanine methyl ester hydrochloride[8] (0.38 g, 2.72 mmol) according to the same procedure as described for the synthesis of **164a**. After irradiation for 23 h the reaction mixture was stored overnight at 4 °C. The precipitate was collected by filtration to give pure **164f** as a white powder (1.08 g; 69.5%). The filtrate was worked up as described for **164a** and the residue was purified by centrifugal chromatography (CH_2Cl_2/EtOAc, 4:1) to give a second crop of **164f** (0.12 g; 7.7%; total yield: 77.2%); mp 150-152 °C.

^{1}H-NMR ($CDCl_3$): 1.20-1.79 (m, 4H, hArg γ- and δ-H), 1.38 (s, 9H, Boc), 2.29 and 2.59 (2x m, 2H, hArg α-H), 2.38 (m overlapping, 2H, Ala β-H), 3.19 and 3.38 (2x m, 2H, Ala β-H), 3.61 (s, 3H, OCH_3), 3.62-3.96 (m, 3H, hArg β- and ε-H), 5.09 and 5.13 (AB-system, *J*=14.7, 2H, $OC\underline{H}_2Ph$), 5.22 (s, 2H, $OC\underline{H}_2Ph$), 5.83 (bd, 1H, *J*=8.4, hArg β-NH), 6.63 (m, 1H, Ala β-NH), 7.28-7.43 (m, 10H, Z aryl), 9.29 and 9.39 (2x bs, 2H, ω- and ω'-NH). ^{13}C-NMR ($CDCl_3$): 24.6 (δ-hArg), 28.2 ($OC(\underline{C}H_3)_3$), 29.9 (γ-hArg), 33.7 (α-Ala), 34.5 (β-Ala), 38.2 (α-hArg), 44.4 (ε-hArg), 47.5 (β-hArg), 51.5 (OCH_3), 66.9 ($O\underline{C}H_2Ph$), 68.8 ($O\underline{C}H_2Ph$), 78.8 ($O\underline{C}(CH_3)_3$), 127.9 (aryl), 128.1 (aryl), 128.2 (aryl), 128.3 (aryl), 128.6 (aryl), 128.7 (aryl), 128.9 (aryl), 134.5 (aryl-1), 136.4 (aryl-1), 155.4 (C(O)O, Z), 155.6 (C(O)O, Boc), 160.4 (C=N), 163.4 (C(O)O, Z), 171.2 and 172.2 ($\underline{C}(O)OCH_3$ and C(O)NH). FAB-HRMS: calcd for $[C_{40}H_{48}N_6O_9 + H]^+$ 642.3138, found 642.3174.

Benzyl 2(*R*)-[[3(*S*)-amino-6-[[imino[(phenylmethoxycarbonyl)amino]methyl](phenylmeth-oxycarbonyl)amino]hexanoyl]amino]-3-phenylpropanoate (165)

Prepared from **164a** (0.79 g, 1.01 mmol) according to the same procedure as described for the preparation of **151**, to give **165** as a thick oil (0.74 g, 100%).

^{1}H-NMR ($CDCl_3$): 1.03-1.57 (m, 6H, hArg γ-, δ-H and NH_2), 2.05 (m, 2H, hArg α-H), 2.85 (m overlapping, 1H, hArg β-H), 2.93 (m, 2H, Phe β-H), 3.80 (m, 2H, hArg ε-H), 4.80 (m, 1H, Phe α-H), 5.04 and 5.12 (AB-system, *J*=13.0, 2H, $OC\underline{H}_2Ph$), 5.09 (s, 2H, $OC\underline{H}_2Ph$), 5.19 (s, 2H, $OC\underline{H}_2Ph$), 6.90-7.00 (m, 2H, aryl Phe), 7.09-7.36 (m, 18H, aryl), 7.85 (bd, *J*=8.1, Phe α-NH), 9.20 and 9.38 (2x bs, 2H, hArg ω- and ω'-NH). ^{13}C-NMR ($CDCl_3$): 24.3 (δ-hArg), 33.9 (γ-hArg), 37.4 (β-Phe), 42.3 (α-hArg), 43.9 (ε-hArg), 47.5 (β-hArg), 52.8 (α-Phe), 66.5 ($O\underline{C}H_2Ph$), 66.6 ($O\underline{C}H_2Ph$), 68.5 ($O\underline{C}H_2Ph$), 126.5 (aryl), 127.5 (aryl), 127.6 (aryl), 128.0 (aryl), 128.1 (aryl), 128.2 (aryl), 128.4 (aryl), 128.5 (aryl), 128.9 (aryl), 134.3 (aryl-1), 135.0 (aryl-1), 135.9 (aryl-1), 136.6 (aryl-1), 155.4 (C(O)O, Z), 160.1 (C=N), 163.4 (C(O)O, Z), 171, 3 and 171.4 (C(O)NH and C(O)OBzl).

Benzyl 2(*R*)-[[3(*S*)-[[[1-(*tert*-butyloxycarbonyl)pyrrolidin-2(*S*)-yl]carbonyl]amino]-6-[[imi-no[(phenylmethoxycarbonyl)amino]methyl](phenylmethoxycarbonyl)amino]hexanoyl]amino]-3-phenylpropanoate (166)

Prepared from **165** (1.20 g, 1.77 mmol) according to the same procedure as described for the synthesis

of **152**. The crude product was purified by centrifugal chromatography (CH_2Cl_2/EtOAc, 4:1), to give **166** as a thick, faintly yellow oil (1.06 g, 68.3%).
^{1}H-NMR (DMSO-d_6): 1.12-1.81 (m, 7H, Pro β- and γ-H, hArg γ- and δ-H), 1.24 and 1.35 (2x bs, 9H, Boc rotam), 1.96 (m, 1H, Pro β-H), 2.21 (bd, *J*=6.6, 2H, hArg α-H), 2.95 (m, 2H, Phe β-H), 3.29 (m, 2H, Pro δ-H), 3.62-4.07 (m, 4H, Pro α-H, hArg β and ε-H), 4.51 (m, 1H, Pro α-H), 5.02 and 5.3 (AB-system, *J*=12.8, 2H, O$\underline{C}H_2$Ph 2x), 5.21 and 5.23 (AB-system, *J*=12.8, 2H, O$\underline{C}H_2$Ph), 7.10-7.48 (m, 20H, aryl), 7.61 (m, 1H, hArg β-NH), 8.40 (bd, *J*=6.9, 1H, Phe α-NH) and 9.15 (bs, 2H, hArg ω- and ω'-NH). ^{13}C-NMR ($CDCl_3$): 23.4 and 24.3 (γ-Pro rotam), 24.3 (δ-hArg), 29.2 (γ-hArg), 29.3 and 31.1 (β-Pro rotam), 36.4 and 37.4 (α-hArg rotam), 37.3 (β-Phe), 44.2 (ε-hArg), 45.7 and 46.0 (β-hArg rotam), 46.8 (b, δ-Pro), 52.7 (b, α-Pro), 60.4 and 61.0 (α-Pro rotam), 66.6 (O$\underline{C}H_2$Ph), 66.9 (O$\underline{C}H_2$Ph), 68.7 (O$\underline{C}H_2$Ph), 79.8 (C(O)O$\underline{C}(CH_3)_3$), 126.6 (aryl), 127.2 (aryl), 127.9 (aryl), 128.2 (aryl), 128.3 (aryl), 128.3 (aryl), 128.4 (aryl), 128.7 (aryl), 128.7 (aryl), 128.8 (aryl), 129.1 (aryl), 134.6 (aryl-1), 135.1 (aryl-1), 135.9 (b, aryl-1), 136.7 (aryl-1), 154.4 and 154.8 (C(O)O, Boc rotam), 155.6 (C(O)O, Z), 160.3 (C=N), 163.3 (C(O)O, Z), 171, 2 and 171.3 (C(O)NH and C(O)OBzl) and 171.7 (b, C(O)NH Pro-hArg).

2(*R*)-[[3(*S*)-[[[1-(*tert*-butyloxycarbonyl)pyrrolidin-2(*S*)-yl]carbonyl]amino]-6-[[imino(amino)-methyl]amino]hexanoyl]amino]-3-phenylpropanoic acid hydrochloride (167)
Prepared from **166** (1.00 g, 1.14 mmol) according to the same procedure (at 0 °C) as described for the preparation of **68** [Chapter Three], to give **167** as a white foam (0.59 g, 93.3%).
^{1}H-NMR (D_2O): 1.28-1.90 (m, 7H, Pro β- and γ-H, hArg γ- and δ-H), 1.31 and 1.34 (2x bs, 9H, Boc rotam), 2.09 (m, 1H, Pro β-H), 2.34 (bd, *J*=6.7, 2H, hArg α-H), 2.92 and 3.21 (2x m, 2H, Phe β-H), 3.09 (m, 2H, hArg ε-H), 3.37 (m, 2H, Pro δ-H), 3.91-4.19 (m, 2H, hArg β-H, Phe α-H), 4.55 (m, 1H, Pro α-H) and 7.15-7.40 (m, 5H, Phe aryl). FAB-HRMS: calcd for $[C_{51}H_{73}N_9O_{12} + H]^+$ 533.3088, found 533.3078.

A.6. References and Notes

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6. Podlech, J.; Seebach, D. *Angew. Chem.* **1995**, *107*, 507; Podlech, J.; Seebach, D. *Liebigs. Ann.* **1995**, 1217.

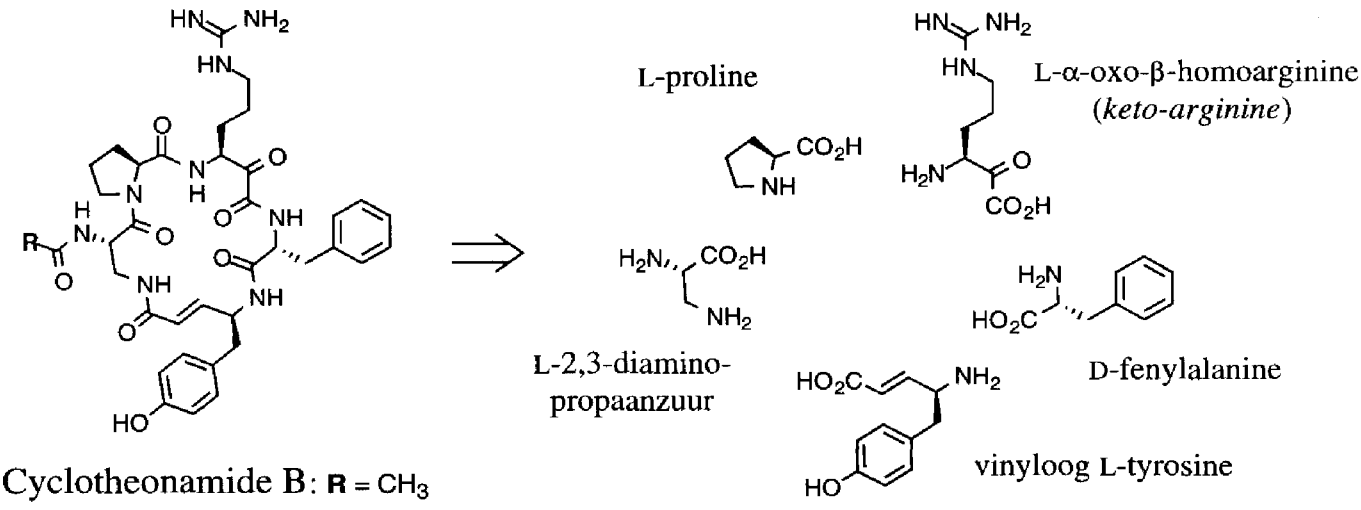
7. D-Phenylalanine benzyl ester hydrochloride was prepared from D-phenylalanine and benzyl alcohol according to the same procedure as described for the preparation of D-phenylalanine allyl ester hydrochloride (**137**) in Chapter Six.
8. Amino methyl ester hydrochlorides were prepared according to the same procedure as described for the preparation of alanine methyl ester hydrochloride (**122**) in Chapter Six.

Samenvatting

Dit proefschrift handelt over de synthese en eigenschappen van Cyclotheonamide B [Schema 1], een cyclisch pentapeptide dat aan het eind van de jaren tachtig geïsoleerd werd uit een zeespons. Cyclotheonamide B is een remmer van het enzym trombine dat bij de bloedstolling een centrale rol speelt.

In *Hoofdstuk Eén* wordt een beknopte uiteenzetting gegeven over de fibrine-cascade en fibrinolyse, processen die het stollingsvermogen van het bloed reguleren. Hierbij speelt het enzym trombine (een serine-protease) een belangrijke rol: het zet fibrinogeen om in fibrine, dat vervolgens polymeriseert en zo de basis vormt voor de uiteindelijke bloedprop. In die gevallen dat bloedstolling ongewenst is (trombose) kan remming van trombine een oplossing bieden.
Er zijn verschillende trombine-remmers te onderscheiden: indirecte remmers, die geen rechtstreekse interactie met het enzym aangaan, en directe remmers, die met het natuurlijke substraat (fibrinogeen) concurreren om een plaats in de actieve holte van trombine. Een nieuwe vertegenwoordiger van deze laatste klasse remmers is Cyclotheonamide B, een zeer sterke trombineremmer met een dissociatieconstante van 1.0 nM. De macrocyclische verbinding is opgebouwd uit vijf aminozuren [Schema 1] waarvan alleen L-proline tot de z.g. natuurlijke aminozuren behoort. Het vinyloge tyrosine en het keto-arginine waren tot vóór de isolatie van Cyclotheonamide nog niet eerder aangetroffen in de natuur.

Schema 1. Cyclotheonamide B en zijn aminozuurbouwstenen.



Cyclotheonamide B: **R** = CH_3

Op basis van de structuur veronderstelden wij dat de hoge affiniteit van Cyclotheonamide voor trombine veroorzaakt werd door een covalente interactie van de electron-deficiënte carbonylgroep van de keto-arginine-eenheid van Cyclotheonamide met een serine –OH van het enzym. Deze veronderstelling werd gaandeweg onze werkzaamheden bevestigd door de kristalstructuur-analyse van het trombine-Cyclotheonamide A (**R**= H) complex door Tulinsky.

Cyclotheonamide biedt de mogelijkheid een nieuwe klasse van keto-arginine-bevattende trombineremmers te ontwikkelen. De cyclische structuur, gecombineerd met de aanwezigheid van vier onnatuurlijke aminozuren, zou tevens een gunstig effect kunnen hebben op de biobeschikbaarheid en de biostabiliteit. Nadelig is dat Cyclotheonamide B behalve trombine ook andere, aan trombine verwante,

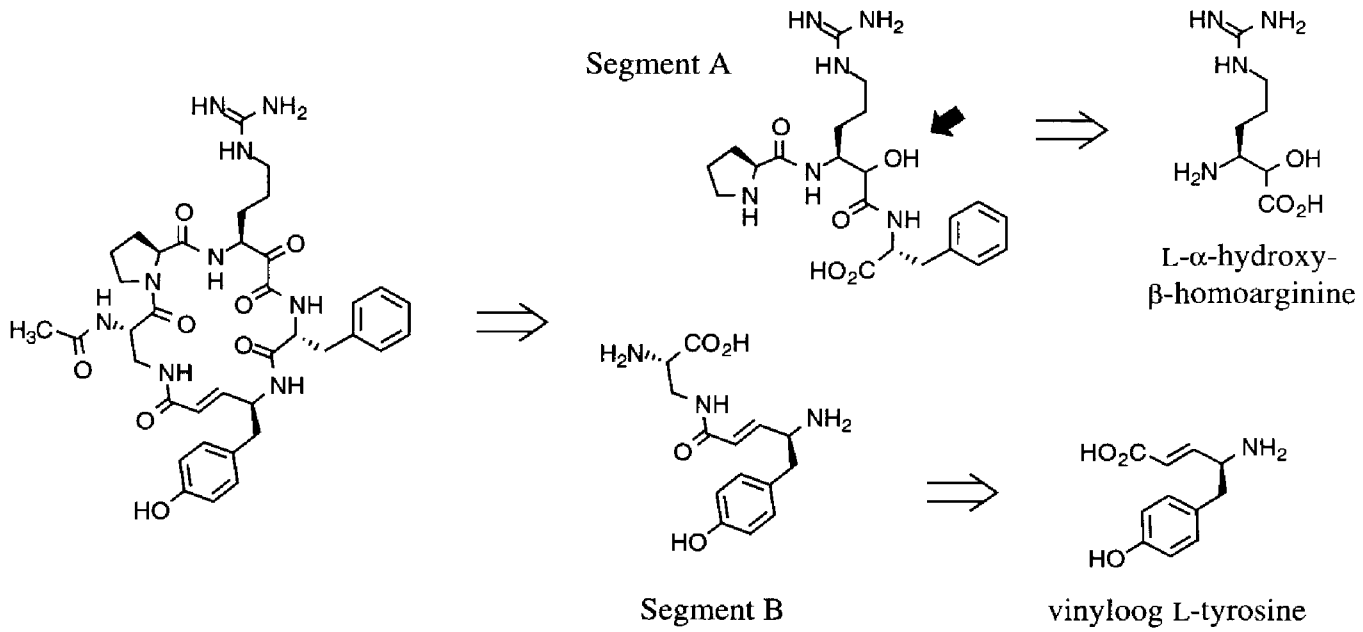
serine-proteasen remt. De synthese die wij wilden ontwikkelen zou daarom niet alleen tot de natuurstof moeten leiden, maar ook bruikbaar moeten zijn om op betrekkelijk eenvoudige wijze analoga te bereiden, opdat de structuur-activiteits-relatie voor trombine-remming zou kunnen worden vastgesteld.

In *Hoofdstuk Twee* wordt een syntheseplan voor Cyclotheonamide B en derivaten opgesteld. Uitgangspunt hierbij is een retrosynthetische analyse waarbij, naast de gebruikelijke praktische aspecten zoals b.v. de beschikbaarheid van uitgangsstoffen, ook theoretische aspecten werden meegenomen. Molecular modelling studies (de kristalstructuur van het trombine-Cyclotheonamide complex was nog niet bekend!) leerden ons dat waarschijnlijk alleen proline, keto-arginine en D-fenylalanine betrokken zijn bij de interactie met het enzym. Opvallend was verder dat de hydrofobe aryl-bindingsholte van het enzym leeg is; de hydrofiele acetylgroep aan de α-aminogroep van 2,3-diaminopropaanzuur is niet in staat deze holte op te vullen. Besloten werd een convergente synthese-strategie te volgen en gebruik te maken van twee fragmenten [Schema 2]: Segment A, een tripeptide bestaande uit de residuen die een interactie met het enzym hebben, en Segment B, een dipeptide dat bij de synthese van analoga gevarieerd zou kunnen worden, om zo de interactie met het enzym te kunnen optimaliseren. Introductie van een hydrofobe groep aan de α-aminogroep van 2,3-diaminopropaanzuur was hierbij een optie.

Omdat het keto-arginine residu zeer gevoelig is voor nucleofielen, werd tevens besloten dat deze eenheid uit een α-hydroxy-β-homoarginine-residu zou worden gegenereerd, en wel zo laat mogelijk in de synthese.

Voorzien was dat de Segmenten A en B opgebouwd kunnen worden uit de overeenkomstige aminozuren, waarbij voor het vinyloge tyrosine en α-hydroxy-β-homoarginine een synthese ontwikkeld diende te worden die bij voorkeur uitgaat van de commercieel verkrijgbare, corresponderende α-aminozuren.

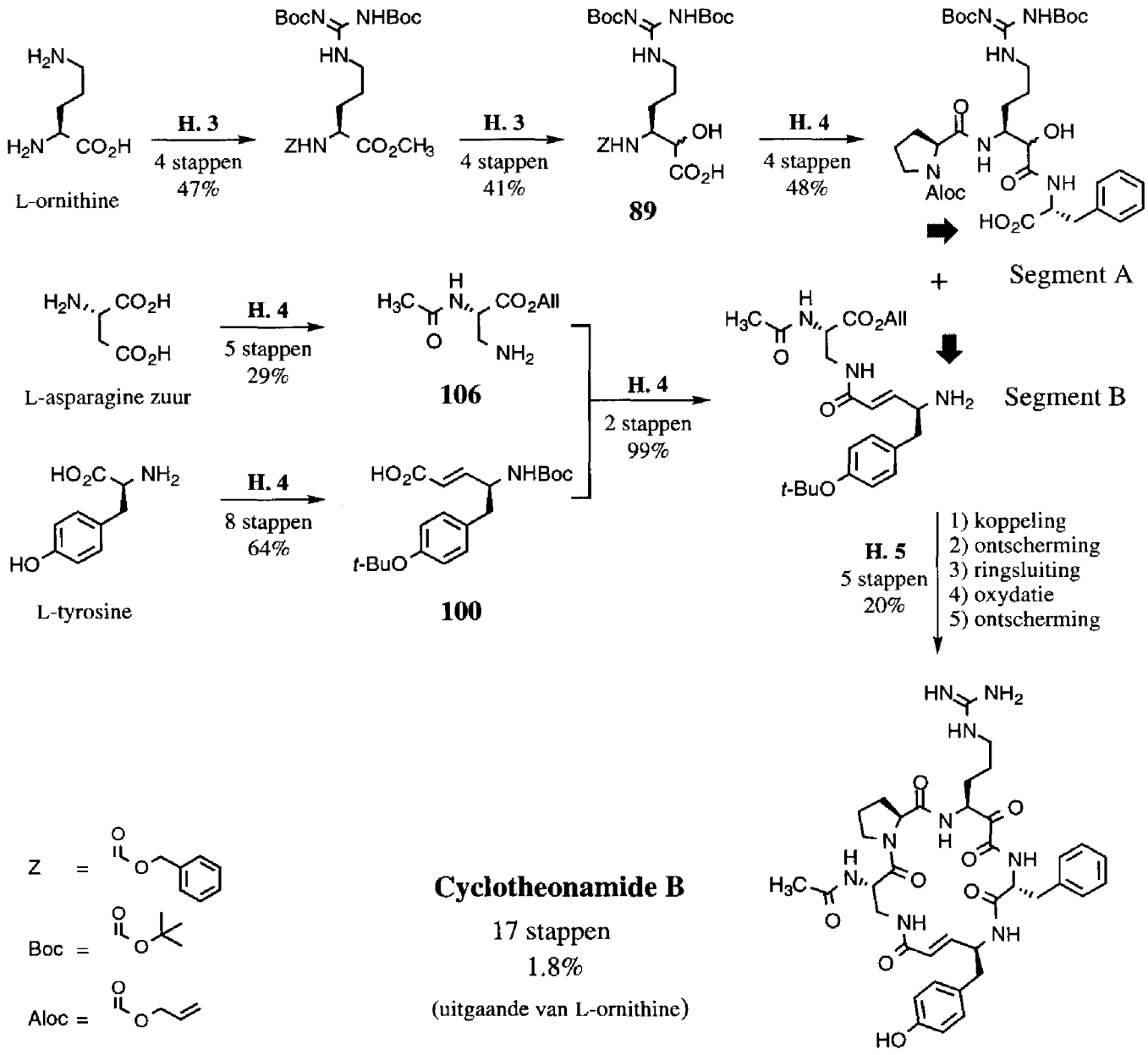
Schema 2. Retrosynthetische analyse van Cyclotheonamide B.



Behalve de definitie van de uitgangsstoffen en intermediairen wordt in Hoofdstuk Twee ook de keuze van beschermgroepen besproken; een keuze die, zoals in de volgende hoofdstukken zal blijken, van cruciaal belang is voor een efficiënte totaalsynthese van Cyclotheonamide B en analoga.

In de *Hoofdstukken Drie* tot en met *Zes* wordt de synthese van Cyclotheonamide B en analoga besproken. In Schema 3 staat de gevolgde route naar Cyclotheonamide B beknopt afgebeeld, waarbij alleen de uitgangsstoffen en enkele belangrijke intermediairen zijn weergegeven.

Schema 3. De synthese van Cyclotheonamide B.



Hoofdstuk Drie behandelt de synthese en eigenschappen van α-hydroxy-β-homoarginine, het synthetisch meest uitdagende deel van Segment A. Zoals verwacht was de omzetting van arginine in zijn α-hydroxy-homoloog geen gemakkelijke opgave. Zowel de oorspronkelijk gekozen Z-beschermgroepen aan de guanidine-eenheid alsmede verschillende beschermgroepen aan de aminogroep van (β-homo)arginine bleken onverenigbaar met tal van reactie-omstandigheden. Uiteindelijk werden twee arginine-homologen bereid (waaronder **89**, Schema 3), beide met een onbeschermde carboxylgroep, een onbeschermde hydroxylgroep, een β-amino beschermgroep die verwijderbaar is in neutraal milieu

en twee guanidine-beschermgroepen die zuurgevoelig zijn. De aanwezigheid en aard van deze laatste beschermgroepen bleek essentieel te zijn voor een succesvolle oxydatie van de α-hydroxylgroep gevolgd door ontscherming van de guanidine-eenheid.

Hoofdstuk Vier beschrijft de synthese van de Segmenten A en B. Van de beide beschikbare α-hydroxy-β-homoarginine-derivaten, beschreven in Hoofdstuk Drie, bleek **89** een geschikte bouwsteen te zijn voor Segment A. De beide aminozuurbouwstenen, benodigd voor de synthese van Segment B, waren betrekkelijk eenvoudig toegankelijk uit α-aminozuren. 2,3-Diaminopropaanzuur werd bereid middels een Curtius-omlegging uit L-asparaginezuur; selectieve invoering van de benodigde beschermgroepen gaf vervolgens derivaat **106**. Het vinyloge tyrosine-derivaat **100** werd bereid uit een beschermd tyrosine-aldehyde *via* een Wadsworth-Emmons olefinering. Koppeling van de aminozuurderivaten **100** en **106** gaf een volledig beschermd dipeptide, dat door middel van selectieve afsplitsing van de Boc groep werd omgezet in Segment B.

Hoofdstuk Vijf beschrijft de synthese van Cyclotheonamide B uitgaande van de Segmenten A en B. Koppeling van deze fragmenten, ontscherming van de uiteinden, ringsluiting, oxydatie van de hydroxylgroep van de homoarginine-eenheid en uiteindelijk ontscherming van de guanidine- en hydroxybenzylgroep verliep zeer efficiënt en gaf synthetisch Cyclotheonamide B, dat volledig identiek is aan het natuurlijke Cyclotheonamide B.

In *Hoofdstuk Zes* wordt de synthese van vier analoga beschreven, gebruikmakend van de route die voor Cyclotheonamide B ontwikkeld werd. Om de op basis van de kristalstructuur gepostuleerde interactie van trombine met de hydroxybenzylgroep te kunnnen verifiëren werd een analogon gemaakt waarbij deze groep vervangen is door een methylgroep. In twee analoga werd een hydrofobe groep geïntroduceerd. In het vierde analogon werd de arginine-zijketen vervangen door een lysine-zijketen. In de synthese van deze analoga was er één opmerkelijk verschil t.o.v. de synthese van de natuurstof, namelijk, de nogal wisselende opbrengst van de ringsluitingsreactie; deze lijkt afhankelijk van het aantal aromatische groepen dat aanwezig is in het lineaire peptide. Verder blijkt een analogon met een kleinere ring (18-atomen) minder stabiel te zijn dan Cyclotheonamide en de analoga met een 19-ring.

Hoofdstuk Zeven bespreekt de resultaten van de biologische testen. De *slow-tight-binding* eigenschappen die voor remming van trombine door natuurlijk Cyclotheonamide A zijn beschreven werden ook waargenomen voor synthetisch Cyclotheonamide B.
Opmerkelijker zijn echter de anti-trombine-activiteiten van de vier analoga. Zowel de vervanging van de hydroxybenzylgroep door een methylgroep, als ook de introductie van een grote hydrofobe groep aan de α-aminogroep van het diaminopropaanzuur-residu veroorzaakten geen grote verandering in anti-trombine-activiteit. Vervanging van de arginine-zijketen door een lysine-zijketen leidde tot een 50-voudige afname van de activiteit. Ook met betrekking tot de selectiviteit ten opzichte van andere serine-proteasen bleken de analoga niet sterk af te wijken van Cyclotheonamide B.

In *Hoofdstuk Acht* wordt onze synthese-route samengevat en vergeleken met de reeds gepubliceerde routes. De biologische gegevens van Cyclotheonamide B en de in dit proefschrift beschreven analoga worden geëvalueerd en tot slot worden structuur-voorstellen gedaan voor tweede-generatie analoga die, naar verwachting, eveneens gemakkelijk toegankelijk zullen zijn *via* de in dit proefschrift beschreven synthese-route.

De *Appendix* beschrijft een nieuwe en snelle methode voor de bereiding van dipeptiden met een *N*-terminaal β-aminozuur, gebruikmakend van de klassieke Arndt-Eistert synthese.

List of Abbreviations

Aa	amino acid
Ac	acetyl
Ala	alanine
Adoc	1-adamantoxycarbonyl
Adoc-Cl	1-adamantyl chloroformate
Adoc-F	1-adamantyl fluoroformate
All	allyl
Aloc	allyloxycarbonyl
Aloc-Cl	allyl chloroformate
APPA	4-amidinophenylpyruvic acid
ar	aryl
Arg	arginine
Asn	aspargine
Asp	aspartic acid
b	broadened
Boc	*tert*-butyloxycarbonyl
Boc_2O	di-*tert*-butyl dicarbonate
Bzl	benzyl
calcd	calculated
Cl_2Bzl	2,6-dichlorobenzyl
COSY	correlated spectroscopy
CtB	Cyclotheonamide B
d	doublet
DCC	dicyclohexylcarbodiimide
dd	double doublet
ddt	doublets of double triplet
DEPT	distortionless enhancement by polarization transfer
diast	diastereomers
DiBAH	diisobutylaluminum hydride
DiPEA	diisopropyl ethylamine
DMAP	4-(dimethylamino)pyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
Dpr	2,3-diaminopropanoic acid
EDC	1-ethyl-3-[3'-(dimethylamino)propyl] carbodiimide
EDTA	ethylenediaminetetraacetic acid tetrasodium salt hydrate
equiv	equivalent(s)
Et	ethyl
Et_2O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethanol
FAB	fast atom bombardment
Fmoc	fluorenylmethoxycarbonyl
GC	gas chromatography
Gly	glycine
hArg	β-homoarginine
His	histidine
h	hour(s)
hLys	β-homolysine
HOAc	acetic acid
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectroscopy
Hz	herz
IC_{50}	concentration that inhibits 50% of enzyme activity
Ile	isoleucine
kArg	keto-arginine (α-oxo-β-homoarginine)
K_i	dissociation constant
kLys	keto-lysine (α-oxo-β-homolysine)
LC	liquid chromatography
Leu	leucine
Lys	lysine
m	multiplet
Me	methyl
MeCN	acetonitril
MeOH	methanol
min	minutes
mp	melting point
MPLC	medium pressure liquid chromatography
MS	mass spectroscopy
Mtr	2,5,6-trimethyl-methoxybenzenesulfonyl
m/z	mass to charge ratio
NEM	*N*-ethyl morpholine
NMR	nuclear magnetic resonance
Pac	phenylacetyl
PE	petroleum ether 40-60
Pfp	pentafluorophenyl
Pht	phtaloyl
Pip	pipecolic acid
PPACK	D-Phe–Pro–Arg-CH_2Cl
ppm	parts per million
Pro	proline
p-TsOH	paratoluenesulfonic acid
q	quartet
rotam	rotamers
rt	room temperature
s	singlet
SEM	[2-(trimethylsilyl)ethoxy]carbonyl
Ser	serine
t	triplet
TBAB	tetrabutylammonium bromide
TBAC	tetrabutylammonium chloride
TBAF	tetrabutylammonium fluoride
TBDMS	*tert*-butyl dimethylsilyl
TBTU	2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
t-Bu	*tert*-butyl
t-BuOH	*tert*-butanol
TEA	triethylamine
TEMPO	2,2,6,6-tetramethyl piperidine-1-oxide
Teoc-OPnp	2-(trimethylsilyl) 4-nitrophenyl carbonate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
THP	tetrahydropyranyl
TIPS	trisopropylsilyl
TLC	thin layer chromatography
TMS	trimethylsilyl
TMS-Cl	trimethylsilyl chloride
TMSE	2-(trimethylsilyl)ethyl
TPAP	tetrapropylammonium perruthenate
Trp	tryptophan
Ts	paratoluenesulfonyl
Tyr	tyrosine
Val	valine
vAla	vinylogous alanine
vGly	vinylogous glycine
vLys	vinylogous lysine
vTyr	vinylogous tyrosine
Z	benzyloxycarbonyl
Z-Cl	benzyl chloroformate

List of Publications

- Bastiaans, H.M.M.; Boeijen, A.; de Grauw, M.; van der Baan, J.L.; Ottenheijm, H.C.J.
  Synthesis and Biological Activity of Cyclotheonamide Analogues
  in preparation
- Bastiaans, H.M.M.; van der Baan, J.L.; Ottenheijm, H.C.J.
  Synthesis of Cyclotheonamide B
  in preparation
- Bastiaans, H.M.M.; van der Baan, J.L.; Ottenheijm, H.C.J.
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Curriculum Vitae

Harold Bastiaans was born on August 19, 1966, in Maastricht, The Netherlands. He graduated from secondary school (HAVO followed by VWO) in 1985.
In 1985 he matriculated at the Vrije Universiteit (Faculty of Chemistry) in Amsterdam. At the Department of Organic and Inorganic Chemistry he studied the synthesis and properties of low co-ordinated phosphorus and silicon containing compounds (1988-1989, prof. F. Bickelhaupt). At the department of Medicinal Chemistry he studied the mechanism of glutathione-dependent protection against microsomal peroxidation (1989-1990, prof. A. Bast), and the synthesis and biological activity of substituted 2-amino-1,10-phenanthrolines (1988, prof. H. Timmerman). He recieved his M.Sc, majoring in Chemistry as well as in Medicinal Chemistry, in October and November 1990, respectively.
In November 1990 he started his Ph. D.-research at the Vrije Universiteit on the total synthesis and biological properties of Cyclotheonamide B, a natural peptide-mimic isolated from a marine sponge (prof. H.C.J. Ottenheijm).
After his graduation he will join the group of professor P.A. Wender, Department of Chemistry, Stanford University, California, as a postdoctoral research fellow.

His main interest lies in the field of synthetic organic chemistry, related to biological problems, such as molecular recognition, molecular design and biological action.

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